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QUANTITATIVE THIN-LAYER CHROMATOGRAPHY OF SELECTED PHARMACEUTICALS

A THESIS

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled " Quantitative Thin-Layer Chromatography of Selected Pharmaceuticals ", submitted by James McKay Orr in partial fulfillment of the requirements for the degree of Master of Science.

ABSTRACT

Thin-layer chromatography was employed to separate the components of fourteen selected commercial pharmaceutical mixtures in tablet and capsule form. These components included the amphetamines, certain barbiturates, and several related compounds. This method of separation is accurate, convenient, and allows the chromatograms to be analysed quantitatively. A recording photo-electric densitometer with an electronic integrator was utilized to scan and quantitatively estimate the various constituents which were made visible by specific reagents.

Silica gel G was found to be the adsorbent of choice because of layer stability. A mixture of dioxane-benzene-25 per cent ammonia (40:50:10 v/v) was found to be a suitable developing solvent. This solvent proved universal in application for the separation of the mixtures examined. Most of the drugs studied could be estimated with an accuracy of approximately 5 per cent when applied in concentrations of 25 to 100 micrograms.

Several experimental points are discussed which are necessary for satisfactory quantitative results. It is suggested that the technique constitutes a useful method of micro-analysis with specific application to Pharmacy.

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DEDICATION

This thesis is dedicated to my wife Elizabeth, and our parents, who provided me with unceasing encouragement and understanding during the course of my program.

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PART I

INTRODUCTION

Tablets and capsules provide two of the most convenient forms for the oral administration of drugs. They are easily mass produced, and provide a compact, accurate dose in a readily absorbable form. For this reason, they constitute the largest group of pharmaceutical preparations dispensed. More than 80 per cent of prescribed medication is now " pre-fabricated " by the manufacturer in various dosage forms and tablets and capsules are the method of administration for over 75 per cent of all drugs on prescription.

The growing and wide-spread use of these multi-component dosage forms has given rise to problems in pharmaceutical analysis. In many cases the concentration of an incorporated drug is extremely small - a milligram or less - and rapid techniques of semi-micro analysis have to be devised for assaying the drug. For purposes of quality control, four or more ingredients have to be separated and assayed individually in the finished preparation. Similar problems face law enforcement agencies such as the Food and Drug Directorate which controls the qualitative and quantitative content of manufactured pharmaceuticals. In addition, simple rapid analytical procedures are of great value in toxicological forensic laboratories, where the rapid detection of drugs in biological fluid can be literally a matter of life or death.

Due to the wide variety of drugs used in medicine, an extensive range of tests are necessary for pharmaceutical products. Consequently, pharmaceutical analysis has developed into one specialized area of analytical chemistry and has proved valuable in the field of pharmaceutical production. The influence of analytical chemistry has been necessarily increased to rigorously control the potent drugs now available because as formulation becomes more complex, the greater are the assay problems introduced. Garrett states that the most valuable contribution to the saving of manpower in pharmaceutical laboratories is the replacment of chemical methods by physical techniques and the adaption of these to routine analysis (1).

The fundamental problem in the assay of dosage forms containing two or more drugs is the separation of the components, followed by a procedure which estimates each individually. The initial task, however, is the resolution of the tablet or capsule into the individual constituent drugs. Ideally, such an analytical technique should be simple and rapid while retaining an adequate degree of specificity.

Chromatography is an analytical method for the purification and separation of organic and inorganic substances, and particularly useful for the separation of closely related compounds. For this reason, chromatography is used extensively in pharmaceutical analysis and new techniques are constantly being refined and developed.

Chromatography has opened up new areas of research which could not have been investigated as rapidly with other methods and it has greatly extended scientific knowledge in the fields of biochemistry and drug metabolism.

PART II

LITERATURE SURVEY

History of Chromatography and Application to Analysis

Chromatography is a process for separating the components of a mixture by producing different rates of movement for each component in a two-phase system. It has been defined as the selective adsorption and separation of a mixture of chemical substances on a column or film of adsorbent, through which a suitable solvent has been passed. It is basically a dynamic rather than an equilibrium phenomenon. If one fraction is not specifically retarded relative to the others, no separation occurs. A chemical reaction does not take place during the process and once separated from the mixture, any starting material is recoved unchanged.

The usual type of chromatography involves the movement of a liquid phase over a solid stationary phase which has varying degrees of affinity for the dissolved solutes. Different rates of movement are thus produced for each solute and a physical separation is achieved. The technique has been applied readily to pharmaceuticals including aromatic amines, amino-acids, peptides and proteins, alkaloids, organic acids, phenols, lipids, carbohydrates, antibiotics and antihistamines.

The Russian botanist, Michael Tswett, first described the process in 1903 when he separated plant chlorophylls by filtering a petroleum ether solution of these through a

column of calcium carbonate (2). As the column was developed with petroleum ether, a series of colored bands moved slowly down the column, each band representing a different pigment of chlorophyll. Tswett called the column on which the separation was occurring the chromatogram and the technique the chromatographic method.

Adsorption chromatography, however, was really developed by Kuhn and Lederer (3) and Kuhn, Winterstein and Lederer (4) in 1931 who used the method to separate some polyene pigments into their components of carotene. Great strides were made in carotenoid chemistry in the following years and the use of chromatographic methods contributed significantly to the investigation of leaf pigments, flavines, and other natural pigments. Methods for investigating colorless compounds were devised and column adsorption chromatography rapidly developed into a standard preparative procedure.

Adsorption chromatography is particularly suitable for the resolution of lipophilic substances. Hydrophilic compounds, however, are best separated by the technique of partition chromatography described accurately for the first time by Martin and Synge in 1941 (5). These workers were jointly awarded the Nobel Prize in 1952 in recognition of their studies. Partition chromatography is similar to liquid-liquid extraction procedures in which a chemical mixture is shaken in two immiscible solvents and the individual components separate by virtue of their solubilities in these solvents. On the column, one of the liquids

is present as an adsorbed film on particles of the adsorbent which acts merely as an inert support for the stationary liquid. The second liquid, immiscible with the stationary liquid, is used to develop the chromatogram. The moving liquid is called the mobile phase and the adsorbed liquid on the column the stationary phase. As the column is developed the components of the mixture partition themselves between the two phases. If their partition coefficients differ sufficiently from each other in these solvents the components travel different distances on the column and a separation takes place. Partition chromatography is an extremely efficient method of separating a chemical mixture. Martin and Synge, investigating the amino-acid composition of wool, used silica gel as the adsorbent and a mixture of chloroform and butyl alcohol, saturated with water, as the developing solvent. Water, saturated with chloroform and butyl alcohol, constituted the stationary phase. The mobile phase passed over the stationary phase held in place by the silica gel and the amino-acids were carried down the column at different rates, depending upon their relative solubilities in the organic and aqueous phases. The order of the phases can be reversed in which case the organic solvent is supported by the adsorbent while the aqueous phase travels down the column. This technique is known as reversed-phase partition chromatography. Columns of kieselguhr have been treated with dimethylchlorosilane, a silicone fluid and non-polar organic solvents such as chloroform can then be used as the immobile phase

while the polar aqueous phase is used to develop the column.

Consden, Gorden and Martin replaced the silica gel column by strips of paper and so devised the technique of paper chromatography (6). This is similar in principle to column chromatography, except that the mixture to be resolved is chromatographed on a sheet of filter paper (essentially cellulose) which acts as the adsorbent. The chemical mixture to be separated is applied in a small drop of solution to a strip of filter paper about one inch from the end. When the spot has dried, the paper is allowed to stand in the developing solvent which flows up past the applied spot by capillary action. After the solvent has run its prescribed length, the paper is removed, dried, and the drug or chemical rendered visible or eluted by a suitable method. Paper partition chromatography is achieved by allowing the paper to become saturated with the aqueous phase in an enclosed tank and then developing with the organic phase in the normal manner.

The importance of these techniques in pharmaceutical analysis is illustrated by the fact that four preparations in the British Pharmacopoeia (1963) are assayed by chromatographic methods and eight chromatographic procedures are outlined in the United States Pharmacopeia (Sixteenth Revision).

Chromatographic Procedures in the British Pharmacopoeia (1963) (7)

Assay of Digitoxin (p. 258) and Digitoxin Tablets

The sample of digitoxin, a crystalline glycoside obtained from a suitable species of Digitalis, is assayed by column

chromatography using kieselguhr (siliceous earth) as the adsorbent and benzene-chloroform (3:1 v/v) as the developing solvent. The eluted sample is then evaporated to dryness and, after further treatment, estimated by spectrophotometry.

Assay of Phytomenadione (Vitamin K_1) (p. 614)

The sample of phytomenadione is assayed by column partition chromatography in subdued light using alumina as the adsorbent and trimethylpentane as the stationary phase. The column is developed with anaesthetic ether-trimethylpentane (1:49 v/v) and the eluted phytomenadione is estimated spectrophotometrically at about 249 mμ.

Vitamin A Ester Concentrate (p. 874)

This consists of an ester or a mixture of esters of Vitamin A alcohol in arachis oil or other suitable vegetable oil. The Vitamin A alcohol is estimated by descending paper partition chromatography using dioxane-methyl alcohol-water (70:15:15 v/v) as the mobile phase and liquid paraffin as the stationary phase, the paper being impregnated by saturation with a 10 percent w/v solution of liquid paraffin in light petroleum. The sample is applied in light petroleum and the chromatogram is developed until the solvent front approaches the bottom of the paper. The dried paper is then examined under ultra violet light of long wavelength when the Vitamin A alcohol appears as a fluorescent spot. The fluorescence of any spot corresponding to Vitamin A alcohol in the chromatograms obtained with the con-

centrate is not more intense than that of the spots on the chromatograms obtained with the Vitamin A alcohol.

Related Foreign Steroids (p. 1075)

Related Foreign Steroids are assayed for contamination by descending partition paper chromatography using formamide as the stationary phase and either a saturated solution of formamide in chloroform or a 0.2 percent v/v solution of formamide in a mixture of equal volumes of benzene and chloroform as the mobile phase. After allowing the solvent to approach the bottom of the paper, the paper is dried and then treated with alkaline triphenyltetrazolium chloride solution. The substance being examined and the reference standard each yield, equidistant from the top of the paper, a red spot having the same intensity and order of magnitude. No secondary spots should appear. The developed paper is next examined in ultra violet light of 366 mμ wavelength. The spots obtained with cortisone, hydrocortisone, and fludrocortisone fluoresce; those obtained with dexamethasone, methylprednisolone, prednisolone, and prednisone do not fluoresce.

The British Pharmacopoeia, under Section A of Appendix XIII, gives specific monographs for descending and ascending paper chromatography when used in chromatographic analysis.

Chromatographic Procedures in the United States Pharmacopeia (Sixteenth Revision) (8)

Assay of Digitoxin, Digitoxin Injection, and Digitoxin Tablets (p. 223)

This is carried out with a technique similar to that

described in the B.P.. A column of kieselguhr (siliceous earth) is used to effect the separation of digitoxin in a chloroform-benzene eluate, prior to evaporation and solution in alcohol and subsequent spectrophotometric assay.

Assay of Ouabain and Ouabain Injection (p. 482)

A column of kieselguhr is used to effect the separation of the pure ouabain in an alcohol-chloroform eluate; the solvent is removed by evaporation and the residue dissolved in ethanol. Alkaline picrate reagent is added to develop the color for spectrophotometric assay.

Assay of Sodium Radio-Iodide (I^{131}) Solution for Radio-Chemical Purity (p. 683)

The control solution and the sodium radio-iodide solution are placed on a strip of chromatographic paper and the chromatogram is developed by the ascending technique using 75 percent methanol. The paper is dried and the radioactivity determined in succeeding 1-cm. lengths with a suitable counter. This test is designed to limit radioactivity to iodide and iodate ions with the latter being limited to 5 percent of the total activity.

Assay of Sorbitol Solutions (p. 691)

The sorbitol is separated from solution by adsorption on a column consisting of a kieselguhr mixture which is then developed with dilute isopropyl alcohol. The sorbitol zone is detected by alkaline permanganate and eluted with water after

which it is assayed with potassium periodate.

Assay of Trisulfapyrimidines Suspension and Trisulfapyrimidines Tablets (p. 780)

The individual sulfonamides, sulfadiazine, sulfamerazine, and sulfamethazine, are separated by descending paper chromatography using butanol saturated with aqueous ammonia as the solvent. Following their separation, the sulfonamides are eluted from the paper and treated to allow colorimetric determination.

Assay of Thiamine in Various Mixtures

Although not official, this method involves separation and concentration of the thiamine on a column of purified base-exchange silicate from which it is eluted with hot acid potassium chloride solution. The thiamine in the eluate is then oxidised to thiochrome, extracted with isobutyl alcohol, and then determined by a fluorophotometric method.

Assay of Sterile Phytonadione Emulsion (p. 537)

The phytonadione is isolated from the emulsion by adsorption on a column of alumina which is then developed with n-hexane. The eluate containing the phytonadione is estimated spectrophotometrically.

Assay of Vitamin D (p. 910)

A saponified solution of the vitamin preparation is extracted with solvent hexane and this extract is passed first

through a column of siliceous earth impregnated with iso-octane and polyethylene glycol 600 to remove any Vitamin A present and then through a column of fuller's earth which retains the Vitamin D. The Vitamin D is eluted from the second column with benzene. Antimony trichloride is used to develop a pink color with Vitamin D and this color is evaluated spectrophotometrically.

Under the section of General Information and Procedures, the United States Pharmacopeia defines chromatography as "a procedure by which drug principles and inert materials encountered in pharmaceutical preparations are separated by fractional extraction, or adsorption, or ion exchange on a porous solid, by means of flowing solvents". It then gives monographs on adsorption column chromatography and partition chromatography. It also describes apparatus and procedures for ascending and descending paper chromatography besides discussing the use of reference substances in identity tests and the location of spots on the chromatogram.

Non-Official Chromatographic Procedures

A great many non-official methods have been devised for the analysis of specific pharmaceuticals by chromatography. Levine reported the use of multi-stage columns to separate drug mixtures (9). The separate stages in the columns had either acid or alkali as the immobile or stationary phase and were arranged either as layers in the same column or in separate columns arranged in series. These layers "trap" alkaloidal

or acidic drugs as their water soluble salts from solution in the non-aqueous mobile phase. The separated drugs can be recovered after reversing the pH of the immobile phase. Levine (10) and Koshy (11) used this technique for mixtures of aspirin, caffeine and other drugs while Turi described a similar procedure for the assay of pharmaceutical combinations of aspirin, phenacetin, caffeine and itobarbital with phenothiazine derivatives (12).

Procedures have also been devised employing column and paper chromatography in the assay of steroids, antibiotics, analgesics, barbiturates, Belladonna, Veratraea, Ergot, Rauwolfia and other alkaloids, salts of bases and various other applications (13).

Paper chromatography has two distinct advantages; it is simple to operate and the equipment is relatively inexpensive. Its main disadvantages are the fact that difficulties are encountered when the method is applied to lipophilic substances and that separations can often be slow, ranging from four to twenty hours and even longer.

The latter difficulties have been overcome by the technique of gas chromatography in which substances which are volatile at temperatures below 250°C can be separated by flushing the mixture through a column of inert adsorbent with a carrier gas such as nitrogen. The method is very rapid, the degree of separation or resolution extremely high, and the results can be interpreted quantitatively. Unfor-

tunately, the equiment is expensive and fairly complex and the compound must have some vapor pressure at workable temperatures.

Although the invention of paper chromatography was a major advance in eliminating many of the disadvantages of column chromatography, a convenient micro-method of adsorption chromatography was still lacking. Such a method was devised by the development of thin-layer chromatography (TLC) which consists of spreading a thin layer of adsorbent on a smooth glass surface and resolving an applied mixture with liquid solvents. The method is rapid and the degree of resolution achieved is extremely high. The technique has won wide recognition in many fields since its initial development in 1960, and in the past two years it has been applied increasingly to problems in pharmaceutical analysis. The method lends itself to quantitative interpretation and is also useful as a preparative method.

Thin Layer Chromatography

The first use of thin layers of adsorbent on glass plates was reported in 1938 by Izmailov and Shraiber who described its application to pharmaceutical analysis in the separation of galenicals (14). The authors reported that the substances in the galenical mixture separated into zones when only one drop of the mixture was used. They stated: "The method enables one to obtain satisfactory results using one drop of the substance under test, very small quantities of the adsorbent, and minimal time. The method may be used for the evaluation of galenical preparations and their dentification, as well as for a preliminary test of the adsorbent and the kind of developer."

Three years later Crowe(15) described the use of thin layers of unbound adsorbent to help predict the best solvents for use in column chromatography and Williams (16) in 1947 outlined the procedure of cicular thin-layer chromatography using the adsorbent held between horizontal glass plates. In the same year the use of starch as a binding agent to hold the layers in place was introduced.

Kirchner, Miller, and others published several papers which investigated various adsorbents and experimental techniques and used the method to investigate some terpenoids (17,18). Several other workers also used the method prior to 1956 (19-22).

The technique was placed on a firm scientific and commercial footing by Professor Stahl in Hiedelberg who devised

a system utilizing standard glass plates and adsorbent (Silica gel G) and a new apparatus for preparing the thin layers.(23) Stahl called the method "thin-layer chromatography" or "TLC". Since 1958 the applications of thin-layer chromatography have rapidly increased and it has now become routine method in many laboratories (24,25) .

Besides its use in pharmacy,thin-layer chromatography has several general applications (26). It is often used in a preliminary study of a system or situation such as an initial determination of the components in a plant extract or exploring a large number of possible reaction conditions. It can be used to study intensively a single reaction or to isolate specific reaction products. Quantities less than one gram can be separated by preparative thin-layer chromatography a method which is often much quicker and more precise than the use of column chromatography. The technique can help predict in a short time the best combinations of solvent and adsorbent. The adsorbents used in thin-layer chromatography are so fine (5 millimicrons and smaller) that they cannot be used in column work, but because of its rapidity, thin-layer techniques can be utilized to check the content of the effluent from a column.

The method is being used to an increasing extent in quantitative assay procedures and much of this work finds application in clinical chemistry where speed is of paramount importance. Depending on the technique, the degree of error ranges from one to five percent and higher.

Thin-layer chromatography, like paper chromatography, can be used to determine component patterns for drugs, plant constituents and extracts and biochemical preparations. For rapid qualitative testing these patterns can be compared with adultered samples to give some idea of the extent of contamination, or with reference standards to verify or disprove the presence of specific constituents.

Pharmaceutical analysis is one specialized area of the analytical field. The rapid identification of the active ingredients of medicines is important in pharmaceutical control laboratories and the pharmaceutical chemist should be in a position to determine the composition of currently available medicaments by some simple method. Frequently, color reactions are not specific enough while the techniques necessary for purification and melting point determination are often inadequate and laborious. Rapid and conclusive identifications are possible by thin-layer chromatography.

Similarly, the identification of drugs and poisons in body fluids is of great importance in toxicology. In cases of poisoning by an unknown substance the speed of thin-layer chromatography may be instrumental in saving a life. Two solvents can be sufficient to give a general chromatogram of a large number of drugs and in this way, schemes of toxicological analyses can be devised. Thin-layer chromatography is particulary useful in that corrosive reagents can be applied to chromatoplates but not to paper chromatograms and this can frequently decrease analysis time by speeding color development.

Although there are limitations to its utility (it is a small scale process - quantities greater than 250 micro-grams can overload a plate) the method is an extremely useful chromatographic technique. It is simple, sensitive, and rapid, and the range of compounds which can be treated by the thin-layer technique is very much greater than in any other chromatographic process. Many drugs and groups of drugs have been analysed in specific applications of thin-layer chromatography.

Waldi and others examined the behaviour of more than fifty alkaloids on inorganic layers (27). Each alkaloid had characteristic R_f values in each of the eight solvent systems described and an unknown was identified by making an initial run in two of the solvent systems and then employing further solvents and color producing reagents to detect the alkaloid. Dragendorff's reagent and iodoplatinate reagent are two frequently used sprays used to give typical colors with alkaloids on thin layer plates. The Belladonna alkaloids can be separated on neutral and on alkaline Silica gel G using 70 per cent ethanol and 25 per cent ammonia as the solvent and identified by Dragendorff's reagent (28). A good separation of the most important alkaloids including morphine, codeine, papaverine, and thebaine can be achieved with benzene-methanol on silica gel. Using the same adsorbent with chloroform-ethanol, morphine can be determined quantitatively in opium extracts (29).

Schlemmer and Link determined reserpine and other Rauwolfia alkaloids in medicinal products by thin-layer

chromatography (30). The reserpine was determined by locating the compound on the chromatogram with an ultra-violet lamp, scraping off the silica gel zone quantitatively, eluting with equal volumes of dioxane-90 per cent ethanol and measuring the extinction at 268 millimicrons. A partial separation of the ergot alkaloids on silica gel has also been achieved (31). Good resolution of the purine alkaloids caffeine, theobromine, and theophylline, has been carried out on layers of silica gel buffered to p^H 6.8. Chloroform-96 per cent ethanol was the developing solvent. Antipyrine which is frequently combined with these alkaloids can be detected at the same time using the above solvent system (32).

Some pharmaceutically important amines such as histamine, phenylethylamine, and tryptamine, have also been separated using buffered layers. The amines are usually detected by spraying with ninhydrin. Similarly, adrenaline and noradrenaline can be chromatographed on buffered silica gel using 70 per cent ethanol as the solvent. As little as 0.005 microgram of the compounds can be detected by spraying with a solution of iodine in chloroform or with potassium ferricyanide in phosphate buffer and examining in long-wave ultrviolet light(32). Adrenaline, noradrenaline, serotonin and other catecholamines can also be chromatographed on silica gel after conversion into their acetyl derivatives (33).

Amino-acids can be chromatographed satisfactorily on silica gel. Solvents such as n-butanol-acetic acid-water and phenol-water give very good separations. The limits of detection

by the ninhydrin reaction on silica can be 0.1 microgram or lower (34). Steroids have also been chromatographed on silica gel and on alumina layers and it is claimed that the difficulties encountered with these compounds in paper chromatography because of their poor water solubility are not encountered in thin-layer chromatography. Fokkens and Polderman examined the stability of medicaments containing steriods and the 17-hydroxycorticoids investigated were resolved on layers of calcium sulphate (35).

Cardiac glycosides can be separated on silica gel and a separation of 14 cardiac glycosides has been achieved in thirty minutes with methylene dichloride-methanol-formamide as a solvent system (36). Similarly, the sapogenins digitogenin, gitogenin, digalogenin, and tigogenin have been resolved on silica gel using a chloroform-acetone solvent system; chlorosulfonic acid was the detection agent (37).

Ganshirt and Malzacher (38) contrived a method for separating the water-soluble vitamins aneurine, pyridoxine, nicotinamide, biotin, calcium pantothenate and ascorbic acid in the proporations in which they occur in pharmaceutical preparations. It is generally applicable if about 50 - 100 micrograms of mixture is used containing 1 - 10 micrograms amounts of the B-vitamins and 5 - 30 micrograms of ascorbic acid. The silica gel layer contains 2 per cent phosphor ZS Super and the chromatogram is evaluated in short-wave and long-wave ultra-violet light following development with a benzene-methanol-acetic acid system. Specific color sprays

are used to detect biotin, nicotinamide and calcium pantothenate . Silca gel can also be used to separate nicotinic acid from nicotinamide. Following development, the chromatogram is dried in air and sprayed with methanolic 5 per cent p-aminobenzoic acid solution after which it is exposed to cyanogen chloride vapor prepared in situ. Both vitamins appear as red spots and the amounts can be estimated visually by comparison with a series of known concentrations of the pure substance. This method enables nicotinic acid to be determined selectively in the presence of various other vitamins and admixtures in pharamceutical preparations (39,40).

Davidek and Blattna isolated mixtures of fat-soluble vitamins on layers of alumina. The A-vitamins can be separated from vitamin D_2 using benzene, toluene, xylene, chloroform or carbon tetrachloride. Petroleum ether can be employed to separate vitamin A or vitamin A acetate from β-carotene, or to resolve a mixture of vitamin K_1, vitamin E and beta-carotene. Carbon tetrachloride will separate beta-carotene, vitamin A acetate, alpha-tocopheryl acetate and vitamin D_2. The vitamins are detected by spraying with 70 per cent perchloric acid or 98 per cent sulphuric acid and a variety of colors are produced (41).

As in paper chromatography, antibiotics can be detected on thin-layer chromatograms by the biological acitivity ("bioautography"). After development, the chromatographic plates are coated with a layer of agar containing a small amount of triphenyltetraxolium chloride (TTC) and a micro-organism

which is sensitive to the antibiotic being examined. After some time, light yellow inhibition zones appear in the agar corresponding to the position of the antibiotic on the chromatogram. Where there is no contact with the antibiotic, the agar is colored a deep reddish brown due to the reduction of TTC to triphenylformazen (42). The antibiotics can also be detected by chemical means although this method is less sensitive than microbiological assay. Several penicillins were chromatographed on layers of silica gel by Fischer and Lautner(43) who used acetone-methanol and isopropanol-methanol as solvents. The plates were dried in air and sprayed with N/10 iodine solution containing 3.5 per cent sodium azide. White spots were formed on a brown background and the limits of detection were claimed to be 1 - 2 micrograms. Some preparations give several spots, one of which is often yellow and this provides an additional means of identification. These workers tabulated the Rf values of several commercial penicillins as a potential means of identification. Nussbaumer (44) has demonstrated that some penicillins can be identified by thin-layer chromatography after acid hydrolysis, since the hydrolysis products have markedly different migration rates. Tetracyclines can be detected chemically by spraying the chromatogram with normal hydrochloric acid and heating it at 50°C. for a few minutes. Intensely yellow spots are obtained and the sensitivity ranges from 1.0 microgram to 0.1 microgram. B. subtilis is used as the test organism for microbiological detection. Sharp separations

of rifomycin B. from rifomycin O and of rifomycin S from rifomycin SV have also been achieved (45)and it has been possible to resolve racemic epigriseofulvin from racemic griseofulvin in the naturally occurring antibiotic griseofulvin (46).

The identification of drugs and poisons in body fluids or in tablets is of great importance to the pharmacist. Several workers have devised schemes of toxicological analyses for investigating the purity and nature of unknown drugs. The specimen may be taken from the stomach, blood, cerebrospinal fluid, urine, etc., and is extracted first with ether from a tartaric acid solution and then from an alkaline solution. The extracts are evaporated and then redissolved in methanol after which aliquots are spotted onto the plate. If the presence of morphine derivatives is suspected the acid ethereal solution is made alkaline with ammonium hydroxide and extracted with chloroform under reflux for about 30 minutes. (The extracts are then chromatographed on silica gel.)

Barbiturates and similar compounds are developed with chloroform-acetone while methanol-acetone-triethanolamine is suitable for alkaloids, phenothiazines and substances with similar action. This procedure is said to be of great value for rapid toxicological analysis since the range of compounds which can be separated with these two solvent mixtures is so wide. This considerably reduces the time required to provide the physician with the information needed for treatment.

When a particular compound is suspected it is always advisable to chromatograph an authentic sample on the same plate as a reference standard. The R_f values of barbiturates and similar compounds, alkaloids and basic drugs, opium alkaloids and synthetic substitutes, phenothiazines and compounds with similar activity, and various analgesics extracted from urine at pH 9.0, have been tabulated in various schemes of analyses. Compounds can be extracted both from blood and from homogenized tissue by the methods described and it is not necessary to precipitate proteins. Various basic drugs and narcotics, common in pharmaceuticals, can be separated on silica gel using methanol alone while chloroform-ether is suitable for some hypnotics, analgesics, caffeine, theophylline, and theobromine. Many sulfonamides are readily chromatographed on silica gel and are detected by spraying with ethanolic p-dimethylaminobenzaldehyde in concentrated hydrochloric acid (47,48).

The technique is particularly useful in the analysis of pharmaceutical mixtures. Fifteen common hypnotics (barbiturates and other compounds) are readily separated on silica gel with a mixture of isopropanol-chloroform-25 per cent ammonia. Various metabolic by products can also be detected - for example, the hydrolysis of Librium metabolites by boiling with hydrochloric acid gives 2-amino-5-chlorobenzophenone which can be detected by thin layer chromatography.

The rapid identification of the active ingredients of pharmaceutical preparations such as tablets, capsules,

solutions, ointments, etc., can be carried out fairly readily. Tablets containing aminophenazone, antipyrine, caffeine, and phenacetin were powdered, shaken with 96 per cent ethanol, filtered and made up to 50 ml. with ethanol. Two microliters of the solution are applied to each of two starting points on two separate plates which are then developed with chloroform containing 1 per cent ethanol. After developing for 10 centimeters distance, the first plate is sprayed with aqueous ferric chloride solution (0.5 per cent). A violet spot indicates the aminophenazone and a yellowish-brown spot indicates the antipyrine. The area above the second starting point is sprayed with Dragendorff's reagent and a brick-red spot indicates caffeine. On the second plate the phenacetin is detected by spraying with 12.5 per cent nitric acid and heated at 80 - 90 degrees C. for fifteen minutes which time a yellow spot indicating phenacetin appears (49).

The following procedure has been worked out for tablets containing 50 micrograms caffeine, 200 micrograms phenacetin and
250 micrograms acetylsalicylic acid: Two tablets are finely powered, extracted with 5 ml. methanol and filtered. One microgram of this solution is spotted onto a layer of silica gel containing 2 per cent of the phosphor ZS Super. The chromatogram is then developed with a benzene-ether- acetic acid-methanol system. After developing for about one hour, the three compounds are visible as dark absorption areas under ultra-violet light. The plate can also be sprayed with a freshly prepared solution of ferric chloride-potassium

ferricyanide (one part aqueous 5 per cent potassium ferricyanide, two parts of 10 per cent ferric chloride solution and eight parts by volume distilled water). The acetylsalicylic acid appears as a brown spot and the phenacetin comes up blue. Caffeine can be detected with chloramine-T (50).

Ganshirt and Malzacher devised a simple method for assaying suppositories containing theophylline, papaverine and luminal (50). A suppository is shaken with hot water-methanol until it is completely melted and is then kept in a refrigerator for a few hours until the vehicle has completely solidified. The mixture is centrifuged and 3 microliters of the supernatant liquor is spotted onto a silica gel plate containing a phosphor. The chromatogram is developed for about 90 minutes with benzene-ethanol-acetic acid as the solvent. The drugs are visible as dark absorption areas under ultraviolet light. These workers developed a similar technique for suppositories containing codeine phosphate, aminopyrine, ethyl endomethylenecyclohexenylbarbituric acid, and butazolidine. The suppository was shaken with hot methanol until melted and then placed in a refrigerator until the vehicle has resolidified. Following centrifugation, a few microlitres of the clear supernatant liquor is spotted onto silica gel plates containing a phosphor. The plates are developed for about 90 minutes with benzene-ethanol. All the compounds except codeine phosphate are visible as dark spots under short-wave ultra violet light. If necessary, a plate can be sprayed with alcoholic sodium hydroxide solution which markedly

increases the absorption of the barbituric acid derivative. The plate is then sprayed with iodoplatinate reagent at which time the codeine phosphate forms a violet spot (50).

Thin layer chromatographic methods have been developed for the examination and stability testing of ointments and liniments. In the preliminary treatment, the ointments are dispersed in acetone and the liniments in methanol. The solutions are filtered and a volume of the extract corresponding to about 100 microliters of the mixed active ingredients is spotted onto a silica gel plate containing a phosphor. The plates are developed with a suitable solvent and the drugs detected by ultraviolet light (50).

Many other rapid and definitive identifications have been reported using thin layer chromatographic techniques. Many are extremely specific and all have the advantage of the speed with which such resolutions are carried out (51-54).

PART III

STATEMENT OF THE PROBLEM

Statement Of The Problem

A large number of the pharmaceutical preparations dispensed today contain central nervous system stimulants and depressants often in combination with other ingredients which tend to enhance or aid their effect. It has been estimated that about one half of the prescriptions filled today contain either a central nervous system stimulant, or depressant, either alone or in combination with other drugs. From a survey of the literature, it was found that rapid methods of quantitative analysis do not exist for many of these drug combinations, and it was decided to study a group of commercially available pharmaceutical preparations containing drugs such as amphetamine, meprobamate, certain barbiturates and phenothiazines. Some of the preparations selected contained other ingredients such as acetylsalicylic acid, phenacetin, caffeine, and ephedrine and it was decided that the anaytical method should include these to give a total analysis of the active constituents. In view of its rapid growth in the past few years, thin layer chromatography was selected as the method of choice for reasons of speed, sensitivity, and resolution.

Classification of Selected Drugs

The drugs examined in the selected commercial preparations could be classified as follows:

Analgesics

Acetylsalicylic acid
Phenacetin

Central Nervous System Stimulants

Amphetamine sulphate
Metamphetamine hydrochloride
Theophylline
Caffeine

Central Nervous System Sedatives and Hypnotics

Phenobarbital
Amobarbital
Butobarbital
Pentobarbital
Carbromal

Central Nervous System Tranquillizers

Meprobamate
Prochlorperazine

General Pharmacological Properties Of The Selected Drugs (55-57)

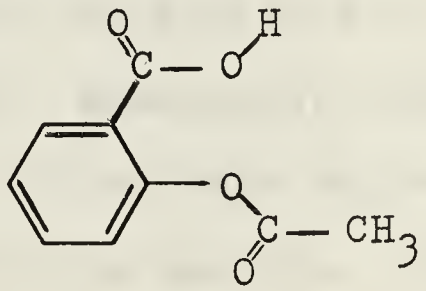
Analgesics

The analgesic (and antipyretic) drugs include a small heterogenous group of compounds which are without addiction liability and are relatively non-toxic. Most of these agents are useful in the treatment of pain and fever and are widely used for minor aches and pains, headaches, and the general feeling of malaise which accompanies such conditions. They are also used to alleviate the symptoms of rheumatic fever, arthritis, gout, and other musculo-skeletal disturbances.

Acetylsalicylic Acid B.P., U.S.P., Ph.I.

$C_9H_8O_4$

M.W. 180.15

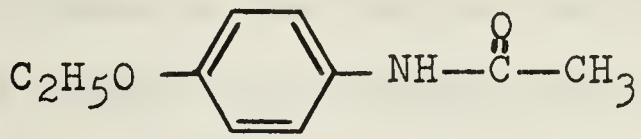


It is employed as an antipyritic and analgesic in a variety of conditions. It does not prevent (or relieve) colds but offers symptomatic relief and is more potent on a weight basis than salicylic acid. It is commonly administered as tablets, capsules, wafers, powders, and suspensions.

Phenacetin (Acetophenetidin) B.P., U.S.P., Ph.I.

$C_{10}H_{13}NO_2$

M.W. 179.22



Phenacetin has the same general usefulness as the salicylates. It is more toxic than the salicylates and cannot be given continuously in large doses. It owes its pharmacological activity to N-acetyl-p-aminophenol to which it is largely altered in the body.

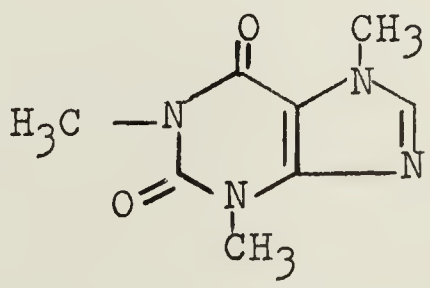
Central Nervous System Stimulants

These drugs increase the activity of some portion of the brain or spinal cord. Xanthines, such as caffeine, act upon the sensory areas in the brain while amphetamine and methamphetamine stimulate the medulla. Since it is not possible to stimulate the central nervous system for a prolonged period of time, these drugs are used normally in the treatment of temporary depressions.

Caffeine B.P., U.S.P., Ph.I.

$C_8H_{10}N_4O_2$

M.W. 194.2

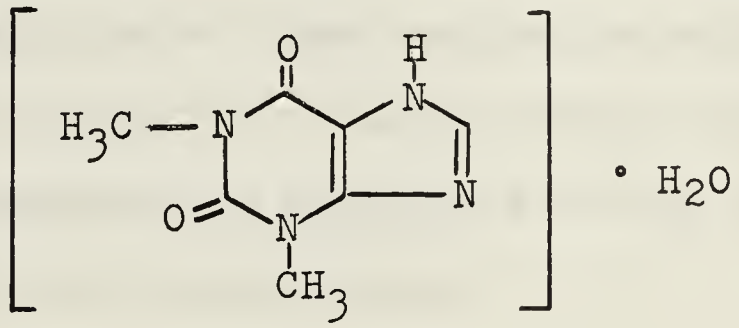


Caffeine belongs to the group of drugs known as the xanthines, the other important members being theobromine and theophylline. They stimulate the central nervous system, increase blood flow through the coronary arteries, relax the smooth muscle of the bronchi, and are active diuretics. Caffeine is often used in combination with analgesic drugs for the relief of headaches, and to counter-act drowsiness. Although not an analgesic itself, it apparently potentiates the action of the analgesics presumably by affecting cerebral circulation.

Theophylline B.P., Ph.I., N.F.

$C_7H_8N_4O_2 \cdot H_2O$

M.W. 198.19

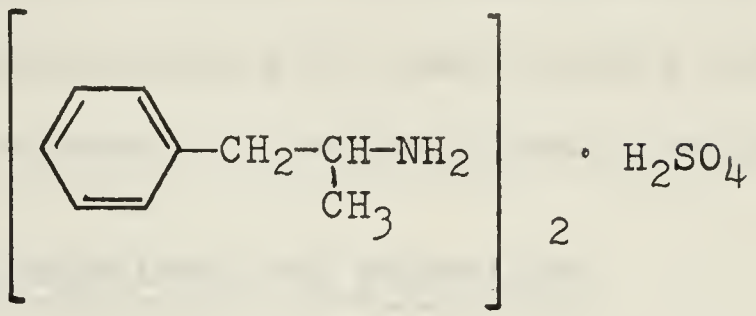


Theophylline is the most widely used drug in the xanthine group. It is an effective diuretic and is used in the treatment of angina pectoris. It is more effective than any other xanthine in relaxing bronchial muscle and is used in cases of asthma.

Amphetamine Sulphate B.P., U.S.P., Ph.I.

$(C_9H_{13}N)_2 \cdot H_2SO_4$

M.W. 368.50

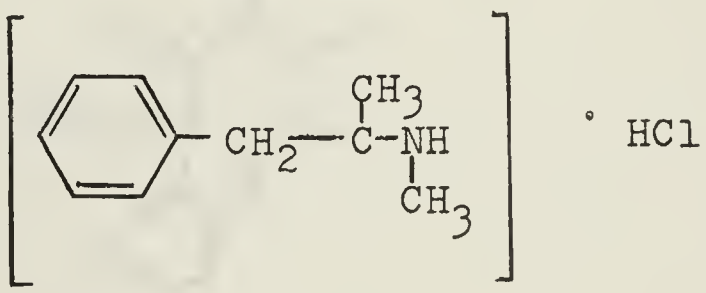


Amphetamine sulphate stimulates smooth muscle and gland cells innervated by sympathetic nerves and has a potent excitatory system on the central nervous system. It constricts small vessels when applied locally and can be used in inhaler form to shrink mucosa in hay fever, acute coryza, vasosmotor rhinitis, and acute sinusitis. It is used in epilepsy with phenobarbital as well as to treat central nervous system poisoning by drugs such as morphine or barbiturates. Aqueous 1 per cent solutions of amphetamine sulphate are used in the conjunctival sac and in the nasal mucosa.

Methamphetamine hydrochloride B.P., N.F., Ph.I.

$C_{10}H_{15}N \cdot HCl$

M.W. 185.70



The uses of methamphetamine hydrochloride are very similar to those of amphetamine. It is more frequently employed for its central nervous system stimulant actions in depressing appetite for the treatment of obesity, in narcolepsy, and in

the treatment of depressant states. It can be administered with agents such as antihistiminics and phenobarbital and may eliminate the sedative effects of these agents without antagonizing the antihistiminic or antiepileptic action.

Central Nervous System Sedatives and Hypnotics

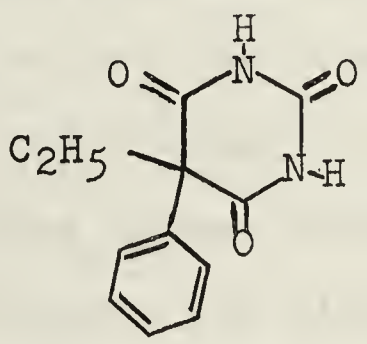
A hypnotic is a drug which induces sleep. A sedative is a drug which induces a milder state of central nervous system depression; that is, the patient may be awake but composed and tranquil. Sedatives and hypnotics differ in the degree of depression they induce in the central nervous system.

The barbiturates are probably the most frequently employed sedative and hypnotic drugs. They are all substituted derivatives of barbituric acid and are classified by the duration of their clinical effects into "long", "intermediate", "short", and "ultra-short" acting barbiturates.

Phenobarbital B.P., Ph.I.

$C_{13}H_{12}N_2O_3$

M.W. 232.24

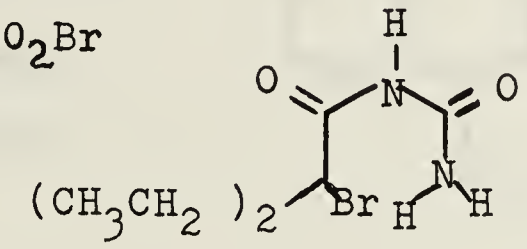


Phenobarbital is a long acting sedative and hypnotic and has specific use in the treatment of epilepsy. The effective dose often produces drowsiness but this can diminished by the concomitant use of amphetamine. With the exception of methobarbital, phenobarbial is the only barbiturate effective in epilepsy. It lacks analgesic properties.

Amobarbital and butabarbital are intermediate acting barbiturates and are used to induce sleep in cases of insomnia. Pentobarbital is a short acting barbiturate often used in combination with other barbiturates.

Carbromal N.F.

$C_7H_{13}N_2O_2Br$



M.W. 237.11

Carbromal is classified as an acyclic ureid which also includes the barbiturates. It is used as a sedative and mild hypnotic in minor forms of insomnia and is considered inferior to the barbiturates. The sleep produced is restful and free from unpleasant after effects.

Central Nervous System Tranquillizers

Tranquillizing agents are used in the treatment of psychoses and neuroses and act on the lower brain areas to produce emotional calmness and relaxation without significant sedation or hypnosis. They reduce anxiety, tension and agitated or disturbed behaviour. One classification divides the drugs into major tranquillizers (for psychoses) and minor tranquillizers (for neuroses).

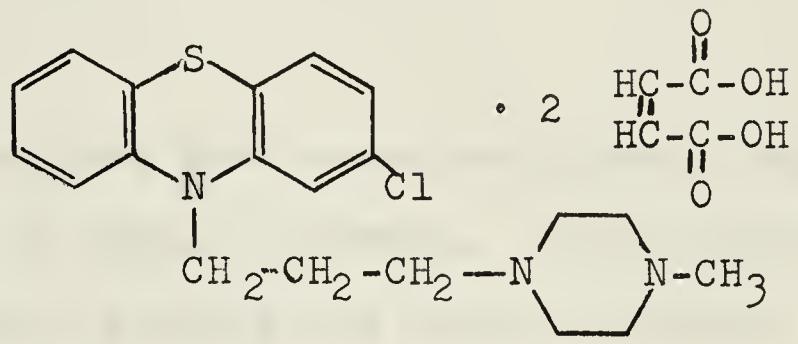
The major tranquillizers include phenothiazine derivaties and Rauwolfia and its alkaloids. These are the drugs of choice in the acutely and chronically disturbed patient.

They are particularly effective in depressive states. There is a fairly high incidence of side reactions but these drugs do not produce habituation or dependence.

Prochlorperazine Maleate U.S.P.

$C_{20}H_{24}ClN_3S \cdot (C_4H_4O_4)_2$

M.W. 606.07

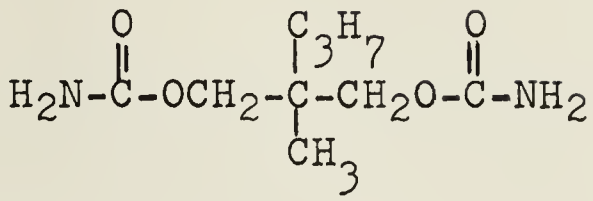


Prochlorperazine is a major tranquillizer of the phenothiazine type. It is used in mild mental disorders in which anxiety, tension, and agitation predominate. It has antiemeticproperties and is used to control mild or severe nausea and vomiting. It can also induce a loss of agressiveness in patients and promote a general calming effect. In the usual therapeutic dose, side effects are generally infrequent.

Tranquillizing agents for neuroses are used in the treatment of transient tension states but are not generally effective in the treatment of disturbed psychotic patients. They produce less "tranquillity" than the major tranquillizers and induce fewer side effects.

Meprobamate B.P., U.S.P.

$C_9H_{18}N_2O_4$



M.W. 218.26

Meprobamate is sometimes used as an antispastic agent and as a mild hypnotic in simple insomnia. It has been employed in psychoneurotic anxiety and tension states, electric shock therapy, and in the treatment of alcoholism. Its over-all toxicity is low but a variety of side effects have been reported.

PART IV

EXPERIMENTAL

Materials And Apparatus

Chemicals

All chemicals and reagents used were American Chemical Society (ACS) or Analytical Reagent grade.

The chemical purity of the standards was checked by observing their melting points and comparing them with the literature. These proved satisfactory in all cases. Each drug produced only one chromatographic spot as further proof of purity.

The following is a list of all drugs used in this investigation:

Acetylsalicylic acid
Phenacetin

d-Amphetamine sulfate
Methamphetamine hydrochloride
Theophylline
Caffeine
Ephedrine
dl-Amphetamine

Phenobarbital
Amobarbital
Butabarbital
Pentobarbital
Carbromal

Meprobamate
Prochlorperazine maleate

Adsorbents

Silica gel G
Cellulose Powder (MN 300)

Solvents

Spotting solvent=Ethanol(95 per cent)
Developing solvent = Dioxane-Benzene-25 per cent Ammonia (40:50:10 v/v)

Spray Reagents

Successful use of the densiometric method depends on the selection of specific staining reagents. Some forty sprays were tried on the drugs involved and of these, twelve were found to be useful in this investigation. The following is a list of the spray reagents used:

1. 50 per cent Sulfuric Acid (aqueous)

The oven-dried plates are sprayed heavily and evenly with the acid. The plates are then placed in an oven at 110-120 degrees C. for 1 hour. The spots are black, brown or grey on a white background and tend to fade slightly after 24 hours. If the plates are over-sprayed, they become quite hygroscopic and will take up enough moisture to obliterate the spots after 1-2 hours. Most drugs can be visualized in this manner, with the exception of some of the barbiturates. Used for: amphetamine phenobarbital, prochlorperazine.

2. Sodium Molybdate (Acid-Molybdate)

0.1 per cent Sodium Molybdate in concentrated Sulfuric Acid

The plates are sprayed lightly with the reagent and then oven-dried at 110-120 degrees for 1 hour. The spots are chocolate brown on a pale grey background. Over spraying will result in the plates becoming hygroscopic, resulting in fading of spots and darkening of background as the plates take up moisture from the air. This stain

works well for the barbiturates which do not come up under the sulfuric acid spray.

Used For: Amobarbital, Butabarbital, Pentobarbital

3. Ferric Chloride Complex

10 per cent Ferric Chloride	2 parts
5 per cent Pot. Ferricyanide	1 part
Distilled water	8 parts

The ingredients are mixed immediately prior to spraying. The plates are sprayed heavily and evenly using a sweeping motion with the sprayer. Color developes to a maximum in about 30 minutes and the spots range from blue to mauve.

Used For: Phenacetin, Acetylsalicylic Acid

4. Tetra-Azotized Benzidine (58)

a. 5 grams Benzidine in 14 ml. concentrated hydrochloric acid then diluted to 1 liter with wate
b. 10 per cent aqueous Sodium Nitrite

The two solutions are mixed together in equal parts before spraying, after which the plates are oven dried for a few minutes at 110 degrees. Amphetamine gives an orange spot on a pale yellow background. The spots tend to fade after 30 minutes.

Used For: Amphetamine

5. Cobalt Nitrate (59)

1.0 per cent Cobalt Nitrate in water

The plates are sprayed heavily and dried in the oven for 15 - 20 minutes. They are then exposed to ammonia

vapor and the spots appear dark blue on a pale blue background. The spots tend to fade after two hours. The barbiturates stain well with this reagent.

Used For: Amobarbital

6. Furfural Spray (60,61)

Furfural 20 per cent in ortho-Phosphoric Acid (v/v)

The reagent is freshly prepared before use. The spots become visible almost immediately after moderate spraying and intensify for a period of about 30 minutes. After this time, they are stable for several days. The spots are black or blue-black on a white to pale grey background. This spray does not require any oven development.

Used For: Meprobamate, Carbromal

7. Mercuric Sulfate-Dithizone (62,63,64)

a. Suspend 5 grams mercuric oxide in 100 ml. water and add 20 ml. concentrated sulfuric acid with mixing. Cool and dilute to 250 ml. with water
b. Diphenylthiocarbazone (Dithizone)

5 grams in 50 ml. chloroform

The plate is sprayed with (a) and, after partial drying, with (b). The spots do not need oven development and range in color from orange to reddish-brown. This spray is most commonly used for barbiturates.

Used For: Phenobarbital, Amobarbital, Pentobarbital

8. Dragendorff's Reagent (Modified) (65,66)

Bismuth Subnitrate	3.4 grams
Glacial Acetic Acid	20.0 ml.

Potassium Iodide 10.0 grams
Distilled water 60.0 ml.

This is a concentrate and must be diluted for use as follows:

1 ml. of above concentrate
3 ml. Glacial Acetic Acid
6 ml. distilled water

The diluted reagent may be kept for 1 week. The plates are sprayed lightly and color developes at once in most cases. The colors range from brick red to yellow.

Used For: Caffeine, Ephedrine

9. Modified Mercuric Sulfate

a. Mercuric SO_4 2 per cent (prepared as before)
b. 0.1 per cent Sodium Molybdate in concentrated sulfuric acid

The plate is sprayed with (a) and then with (b). It is dried for 30 minutes at 110 degrees Centigrade. Certain barbiturates appear as chocolate brown spots on a pale grey background.

Used For: Amobarbital, Butabarbital

10. Modified Sulfuric Acid

a. 50 per cent sulfuric acid
b. 0.1 per cent Dithizone in Ethanol (95 per cent)

The plate is sprayed with (a) until the spots show. Spray (b) is then used. Carbromal yields violet spots on a pink background.

Used For: Carbromal

11. Bratton-Marshall Reagent (67)

a. 1 N Hydrochloric Acid
b. 5 per cent Sodium Nitrite

c. 0.1 per cent solution of N-(1-naphthyl) ethylenediamine di-hydrochloride in ethanol.

The plates are sprayed with (a) and then with (b) until they are transluscent. They are oven dried for a few minutes at 110 degrees Centigrade and sprayed moderately with (c) while still warm. Sulfonamides stain from blue to reddish-purple.

Used For: Sulfonamides

12. Iodine Spray

a. Iodine 5 grams
Potassium Iodide 1 gram
95 per cent Ethanol qs to 100 ml.

b. 25 per cent Hydrochloric acid - ethanol (1:1)

The plate is sprayed with (a) and then with (b). This is an effective spray for theophylline which stains brick red on a grey background.

Used For: Theophylline

Preparation of Thin-Layer Chromatograms

The layers were prepared according to the technique described by Stahl(68,69) who utilizes a spreading device characterised by a moving slurry reservoir and stationary plates (Figure 1).

Five glass plates, 20 x 20 centimeters, were washed thoroughly in chromic acid and water to remove any grease and then were dried. The plates were then placed flush to each other on a plastic aligning tray which has the long retaining edge nearest the operator. The empty spreader was placed on a 5 centimeter starting plate and the silica gel slurry prepared according to the formula:

Silica gel G	30 gm.
1,2-Dimethoxyethane	15 ml.
Distilled water	45 ml.

The silica gel was triturated with the liquid phase until a uniform, homogeneous slurry was obtained. The dimethoxyethane assists in the preparation of films which are smooth and even, with little tendency to crack during storage. It also reduces activation time.

The slurry was poured into the applicator which had been set to produce a layer of silica gel 0.25 millimeters thick. The spreader was pushed evenly along the plates set on the aligning tray to give a uniform thin layer on each plate. The quantity of silica gel prepared in the above formula is sufficient to coat five 20 x 20 centimeter plates to a thickness of 0.25 millimeters.

After allowing the silica gel layer to set (about five minutes), the plates were transferred to a metal drying rack and then activated by heating at 110 degrees Centigrade for for one hour. The plates may then be stored in a dessicator or left in the oven until required.

Preparation of Thin-Layer Plates for Chromatographic Use

A plastic template is laid across one end of a plate and a line drawn through the silica gel with a blunt pencil. This line is usually about one inch from the edge of the plate and marks the limit to be reached by the developing solvent.

The samples for analysis are applied in alcoholic solution at the opposite end of the plate about one inch from the edge. The compounds for analysis were applied from self-filling lambda pipettes calibrated to deliver accurately known volumes. The area of spot application was usually about 6 - 8 millimeters in diameter. The plates were then placed in glass developing tanks (24 centimeters long and high x 12 centimeters wide)(Fig 2) lined with solvent saturated filter paper. Solvent saturation was achieved by allowing the covered tanks to stand for at least 4 hours prior to use.

The plates were allowed to remain in the tanks until the solvent reached the line drawn at the top of the plate (a distance of 15 centimeters). The plates were then removed, dried at room temperature for 15 minutes, oven dried for 20 minutes at 110 degrees centigrade, and sprayed with the

appropriate reagent. The Nutritional Biochemical Corporation* Universal Aerosol Kit (Figure 3) was used to apply the reagent evenly to the plate (Figure 4). The kit consists of a calibrated reagent jar which is attached to a container of Freon propellant. Following the application of the reagent, the R_f values of the compounds were determined and the plates were then ready for quantitative evaluation (Figures 5 and 6).

* Nutritional Biochemical Corporation, Cleveland, Ohio, U.S.A.

Measurement of Spot Area

A Photovolt Densicord Densitometer (Model Number 542) was used to obtain quantitative results from the developed thin-layer chromatograms. This instrument can also be used to scan paper, cellulose acetate, disc, and other gels as well as starch and agar plates. The Densitometer can furnish a plot of the optical absorbance of each spot on the plate and it is therefore possible to obtain direct quantitation of each fraction resulting from resolution of a mixture. This procedure eliminates the need for elution techniques which are often cumbersome and tedious.(Figures 7 and 8)

Theoretical Principles of the Photovolt Densicord 542 (70,71)

The Densicord Model 542 is a complete recording optical densitometer and consists of an electric photometer, an automatic scanning mechanism, and a variable-response recorder. For quantitative calculation, the areas under the curve are recorded by the Densicord Electronic Integrator. The electric photometer is highly sensitive which permits high-resolution scanning with a 0.1 millimeter slit. The high sensitivity makes it possible to measure the transmitted light directly through the opaque layer and averages the light absorbed by the entire spot. At the same time, the evaluation can be made in narrow spectral bands or by fluorescence.

The densitometer operates by using a stabilized light source to provide a beam which is collimated by a cylindrical lens to illuminate the slit evenly. The plate, containing

the stained sample, is positioned on a motor-driven stage which carries the plate under the slit for scanning. The light, attenuated by absorption and scattering due to the sample, falls on the cathode of the phototube.

In plotting the areas of the respective stains, it is necessary to obtain a sweep or excursion of the pen proportional to the concentration of the materials being scanned. Since absorbance is a function of concentration, the electrical impulse has to be logarithmized to yield pen movement in terms of absorbance - for example, twice the concentration should double the pen excursion. However, if the layer being scanned scatters light, the linear relationship between concentration and absorbance (Beer's Law) is no longer valid and deviations from it may become quite large. To overcome this difficulty, the recorder is equipped with an electronic network (Response Control) with eleven positions, one of which will provide the appropriate recording function approximating closely a linear relationship between concentration and adsorbance; i.e., pen excursion proportional to concentration.

The particular response control selected will vary with the absorbent and the spray used to render the spots visible. Consequently, it is necessary to calibrate the instrument by running a set of standards to establish the range over which this linearity is valid. Once established for a specific technique, the setting does not require adjustment unless the adsorbent or spray is altered.

If in the process of staining the spots, the background becomes colored but is still distinguishable from the spots, the use of filters which increase contrast by reduction of the number of wave lengths of light entering the phototube will allow the spots to be evaluated. The selection of a filter is empirical and depends upon the stain used but the filter should be chosen to give the best pen excursion possible while retaining linearity.

If the individual fractions of a drug mixture are complely resolved and the resolution and recorder response setting is optimum, the height of the recorded peaks will be proportional to the concentration of the individual drugs. In practice, however, some degree of "tailing" almost always occurs. In these cases, the basis of quantitation is derived from the area under the curve which must be determined for each peak. These areas can be determined either by counting the squares on the recording paper or by planimetry. In the present study, an automatic integrator was employed for this purpose.

Theoretical Principle of the Integraph Automatic Integrator (72)

The Integraph is an electronic recorder which integrates (or evaluates) the area under the curve automatically and continuously while it is being charted. Besides the electronic circuits, it also contains an electro-mechanical pipping device.

In operation, the Integraph receives a signal voltage

from an outlet on the recorder. This voltage is proportionate to the excursion of the recording pen and to the concentration of the components being scanned. The integraph transforms this voltage into pulses whose frequency is exactly proportional to the voltage and thus to the concentration. The pulses actuate the pipping pen which makes the appropriate number of marking or pips on the edge of the strip-chart in exact alignment with that section of the curve to which the integral refers. The area under the curve is read off the chart by counting the pips between points thus yielding a quantitative result.

For convenience in reading the integrals, each tenth or decade count is marked by a reverse stroke of the pipping pen. For large pen excursions, only the decade pips are counted and their number multiplied by ten. For small pen excursions, the unit pips are counted and added to the decade count. (Figure 9)

This method of measurement surpasses that of the usual mechanical devices such as the ball-and-disc integrators and is more accurate and much less tedious than counting the area under the curve visually or by planimetry.

Operation of the Photovolt Densitometer

The phototube arm is adjusted to clear the thin layer by about six millimeters. With the appropriate controls, the zero density (full light) and the infinite density(dark point) are adjusted to the proper settings. The full light setting is made on an unstained area of the plate and the Integraph is set to the lowest pulse rate possible (one blip every 20 seconds). The plate is aligned such that the spots are centered under the photocell slit and the scan is made by activating the chart drive mechanism and thin-layer stage simultaneously. The density of the spot should not vary excessively over the aperture width.

The spots compared must be about the same size and should be elliptical. Chart speed is set at two inches per minute and the carriage speed at one inch per minute.

Figure 1 Thin-Layer Spreading Apparatus.

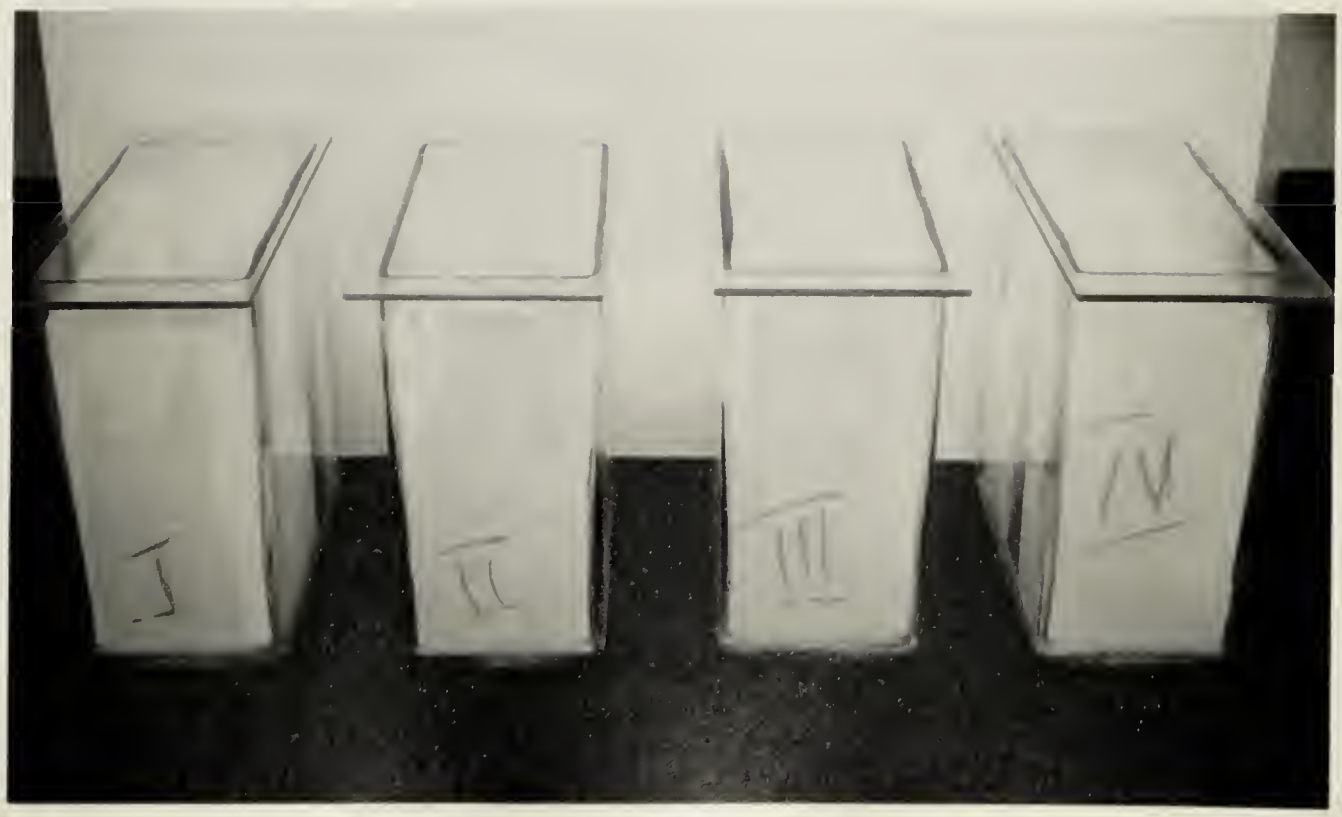


Figure 2 Developing Tanks.

Figure 3 Calibrated Aerosol Sprayer.

Figure 4 Spraying Cabinet Showing Thin-Layer Plate in Position for Spraying.

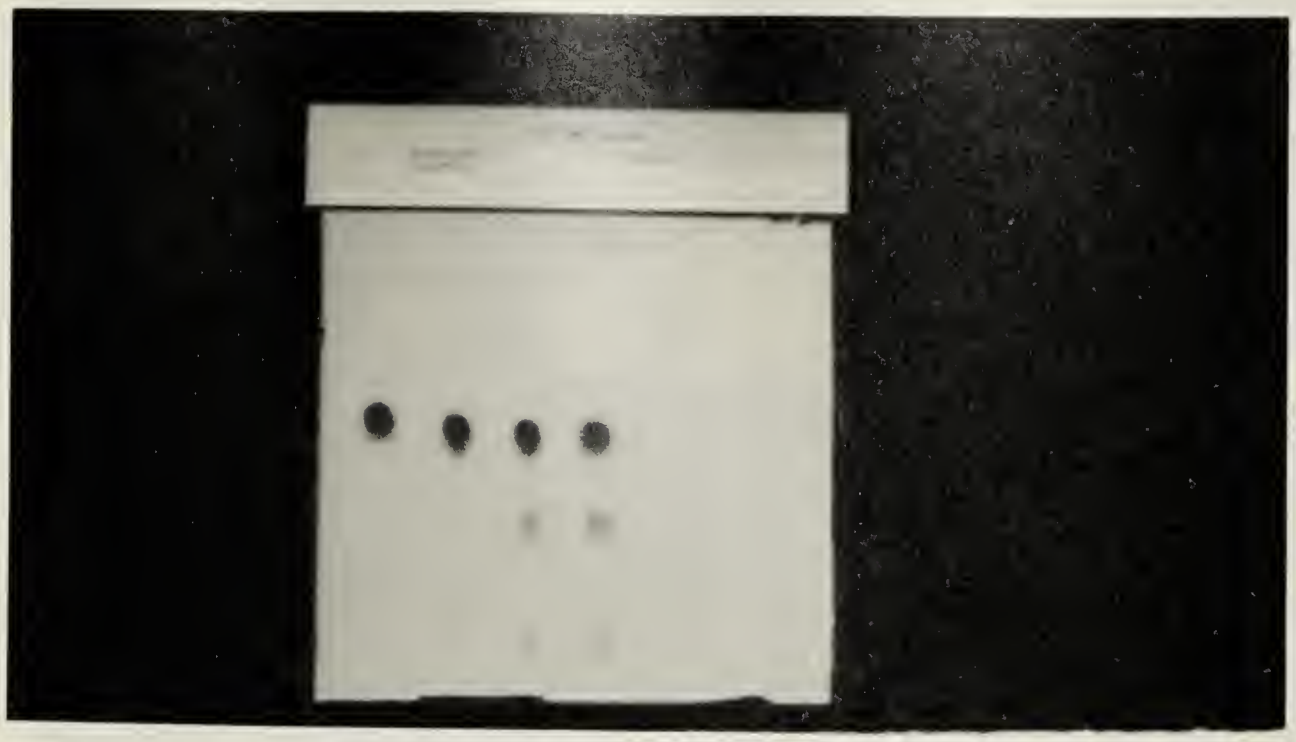


Figure 5 Separation of Phenacetin from Meprobamate in 217-Mep Tablets.

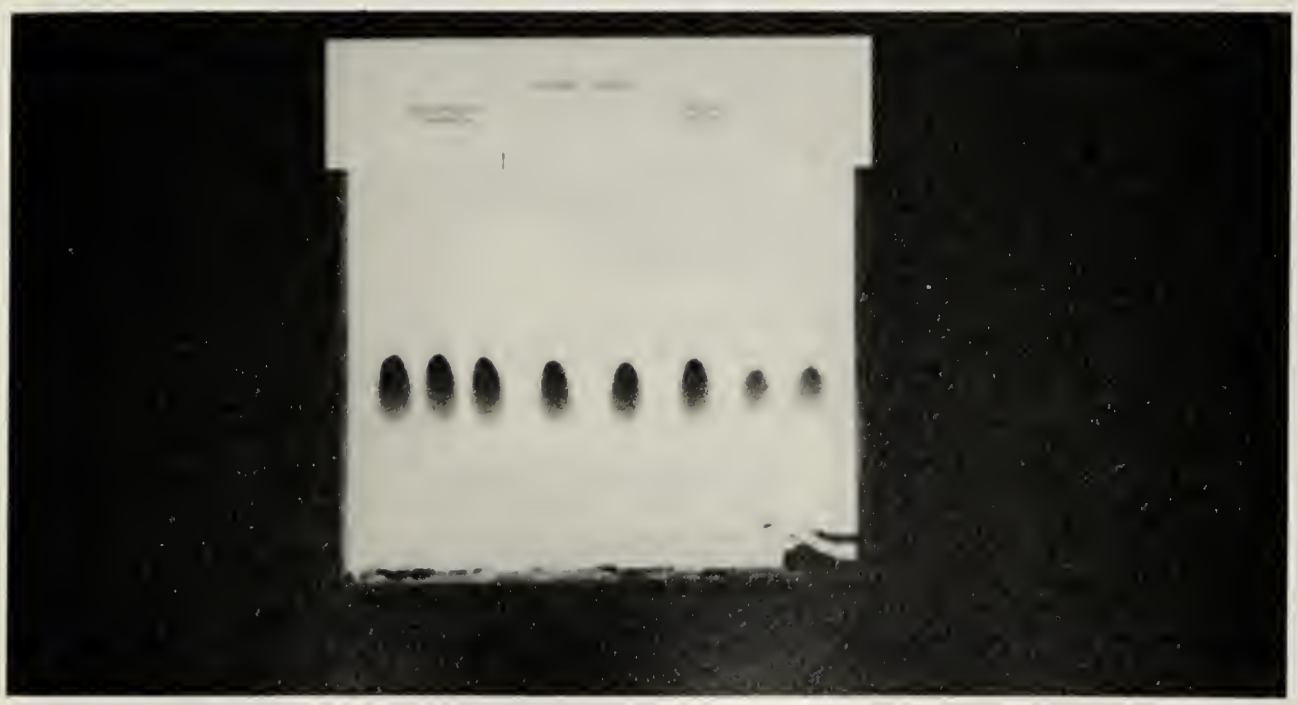


Figure 6 Identification of Meprobamate in Bamadex Tablets.

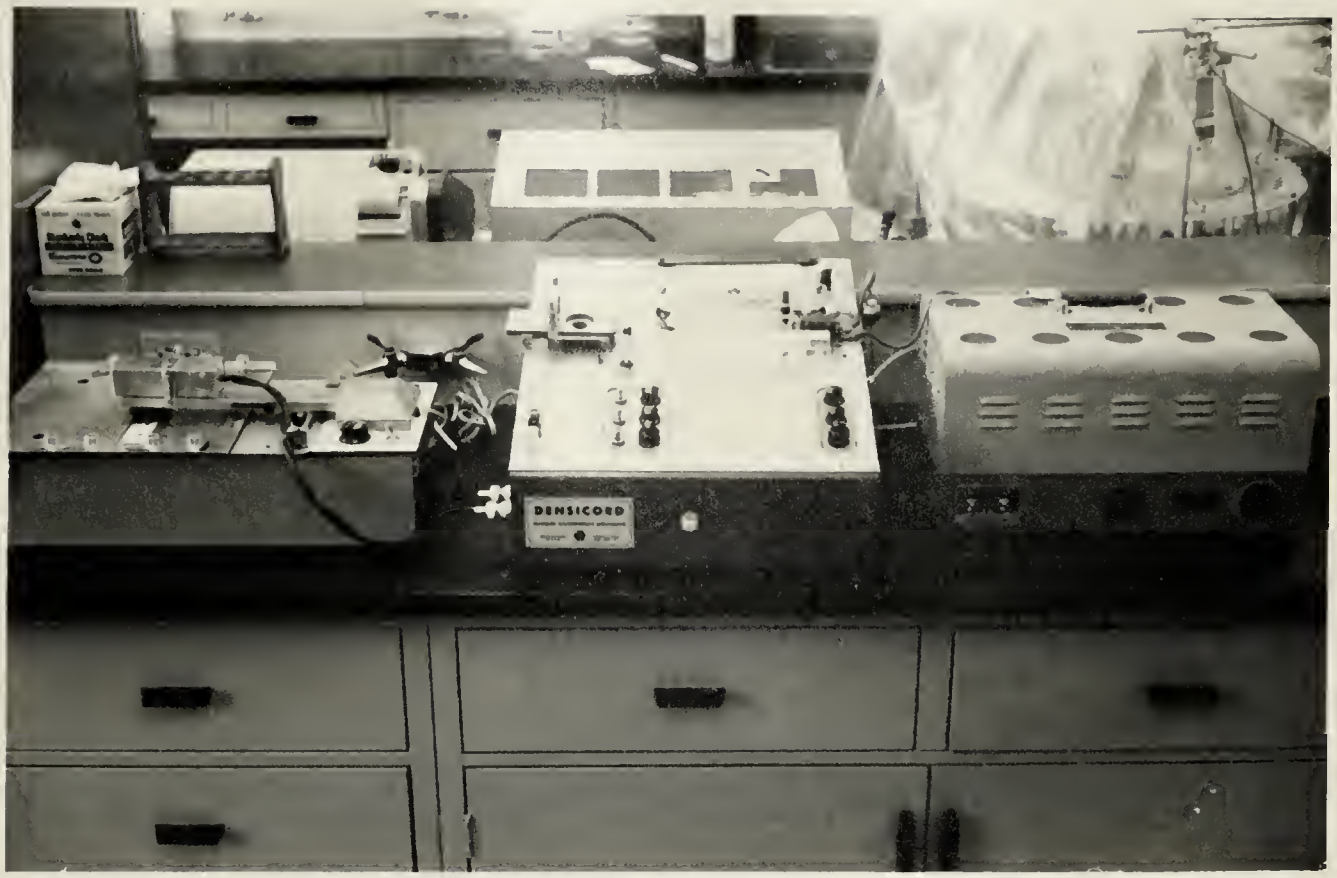


Figure 7 Photovolt Densicord (542) Densitometer with Integraph Attached.

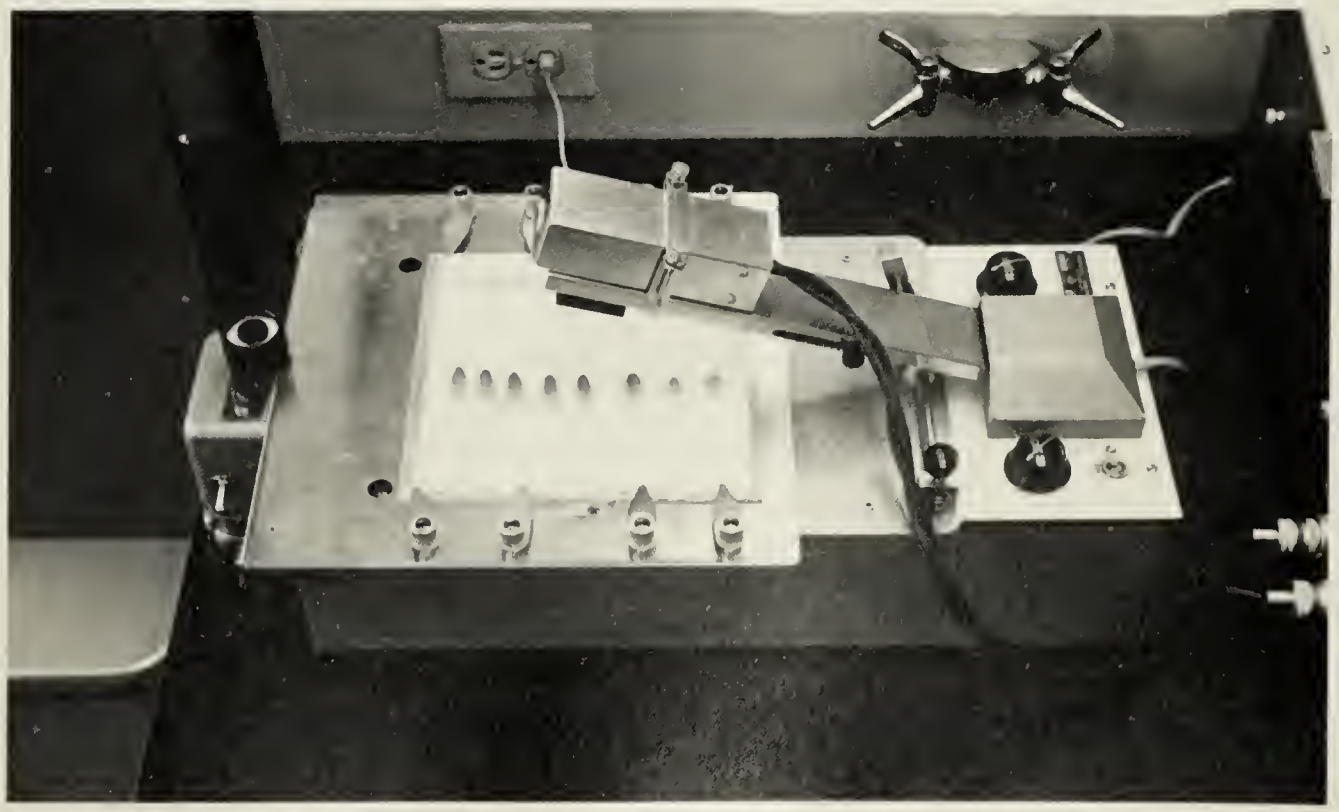


Figure 8 Thin-Layer Stage with Scanning Arm.

Figure 9

Densitometer Chart Record

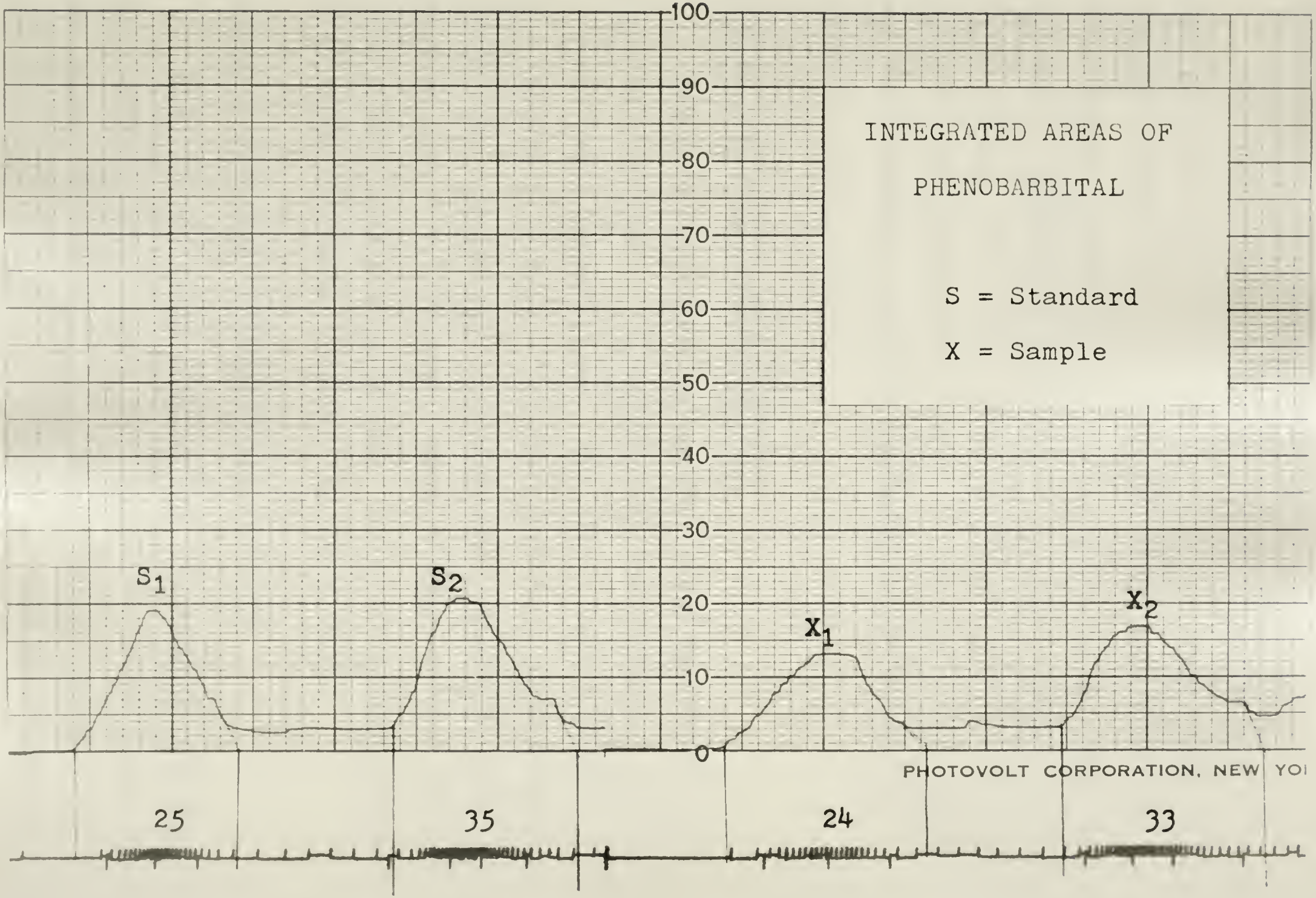
Showing Scanned Integrated Areas

for Phenobarbital

Amount of Standard = 80 micrograms

Response Setting = 5

Filter = None


100
90
80
70
60
50
40
30
20
10
0
INTEGRATED AREAS OF
PHENOBARBITAL
S = Standard
X = Sample
S1
S2
X1
X2
PHOTOVOLT CORPORATION, NEW YOI
25
35
24
33

Preparation of the Standard Curves (Table I)

In order to establish that a relationship exists between spot area and spot concentration for the drugs under study, standard curves for three of these drugs were prepared. Amphetamine, phenobarbital, and acetylsalicylic acid were spotted in various concentrations in their respective detectable ranges. The plates were developed, dried, and the spots sprayed with appropriate reagents. They were then scanned and the integrated areas plotted against concentration. (Figures 10,11, and 12)

Assay of Simulated Drug Mixtures

Ethanolic solutions of several drugs were made up and spotted on plates in concentrations approximating those of certain trade preparations. The plates were developed, dried and sprayed with various stains to produce colored spots which were scanned using the Photovolt Densicord densitometer. Similar spots of individual standard drugs in the same concentrations were chromatographed and the mixtures compared with these standards. (Tables II, IV, V, VI)

Assay of Selected Pharmaceutical Mixtures

Each of the trade preparations was treated individually due to differences in the concentrations of the constituents. The amount of drug applied varied from one product to another but this was necessary to ensure that the spot would at least contain the minimum assayable quantity of the drugs. Another

reason was that certain spray reagents have lower limits of sensitivity which must be exceeded to produce colored spots.

Standards and samples are spotted alternately to facilitate assay and to nullify any variation in the silica gel layer.

The strength and contents of each product, together with other pertinent data, are summarized in the results. (Table III)

1. Bamadex Tablets (Lederle)

Contents:

d-Amphetamine sulfate	5 mg.
Meprobamate	400 mg.

Procedure:

Five Bamadex tablets were shaken with a small amount of water to remove the sugar and color coatings. They were then wiped dry and finely powdered using a mortar and pestle. The powder was mixed with about 20 ml. of ethanol (95 per cent) in a 50 ml. flask and then shaken on a mechanical shaker for 30 minutes. During the shaking time, standards were prepared at strengths equal to the theoretical strengths of the components of the tablet.

The tablet solution was filtered through a Whatman #2 filter to remove the insoluble tablet excipients, and made up to volume in a 25 ml. volumetric flask.

To establish a working range of concentration, quantities from 5 to 25 micrograms of amphetamine and 80 to 1600 micrograms of meprobamate were spotted on a series of plates and the upper and lower levels of detection and separation recorded. The upper limits are dictated by the concentration at which separation becomes impossible due to overloading of one or more ingredients. It was ultimately found that 10 micrograms of amphetamine and 800 micrograms of meprobamate gave consistent, reproducible results.

Ten plates were prepared with at least two spots of the drug mixture on each plate. The appropriate standards were applied to the plate to ensure a total of ten assays for each

drug. They were then developed in the chromatographic tanks for one and one-half hours, while the solvent travelled 15 cm. to a pre-scored line. The plates were then air-dried, oven-dried and sprayed with the appropriate stains to develop visible spots. Furfural stain was used for meprobamate and 50 per cent sulfuric acid for amphetamine. The R_f values were recorded and the plates were scanned by the densitometer. Results are in Table VII of the Appendix.

2. Eskatrol Tablets (Smith, Kline and French)

Contents:

Amphetamine sulfate	5 mg.
Prochlorperazine maleate	2.5 mg.

Procedure:

Five tablets were crushed and powdered in a mortar and pestle and dissolved in 20 ml. of ethanol. Since prochlorperazine is slowly soluble, shaking time was increased to 60 minutes. The solution was filtered through charcoal on a Whatman #2 filter disc and this rinsed with two 2-ml. portions of ethanol. The sample solution was made up to volume with ethanol in a 25 ml. volumetric flask. Standards were prepared in the normal manner and all solutions were stored in brown glass as prochlorperazine is light-sensitive. Limits were established by running a series of several concentrations. It was found that 20 microliters (20 micrograms of amphetamine and 10 micrograms of prochlorperazine) gave the best spots although the recovery of prochlorperazine was not as high as expected.

The sulfuric acid spray stained both drugs, producing violet spots with prochlorperazine and grey on a white background for amphetamine.

Results are in Table VIII of the Appendix.

3. Dexamyl Tablets (Smith, Kline and French)

Contents:

Amphetamine Sulfate	5 mg.
Amobarbital	32 mg.

Procedure:

Five tablets were powdered and mixed with 15 ml. of ethanol followed by 30 minutes of mechanical shaking. A charcoal pad on a Whatman #2 filter disc was used to remove the tablet excipients and coloring matter. The solution was brought up to 20 ml. volume in a previously calibrated flask. Standards were prepared equivalent to the theoretical concentration of the components in the tablet, and upper and lower limits of assay were established. The amobarbital did not color as readily as phenobarbital with the mercuric sulfate-dithizone spray, but developed color well with the cobalt nitrate spray provided the concentration was over 100 micro - grams. After development and spraying, spot size and R_f values were recorded and the plates were then scanned.

Results are in Table IX of the Appendix.

4. Butadex Tablets (Paul Maney)

Contents:

Amphetamine sulfate 5 mg.
Butabarbital sodium 32 mg.

Procedure:

Five tablets were triturated to a fine powder in a mortar and pestle and mixed with about 15 ml. of ethanol. The mixture was shaken mechanically for 30 minutes to assist solution, and the undissolved tablet excipients were removed by suction filtration through a Whatman#2 filter disc. The disc was rinsed with 2 ml. of ethanol and then solution made up to 20 ml. in a calibrated flask. Equivalent standards were also prepared using ethanol. A series of plates had to be developed to determine the range of concentration which allows separation and assay.

A modified spray reagent, containing 2 per cent mercuric sulfate, followed by a counter spray of 0.1 per cent sodium molybdate in concentrated sulfuric acid, produced chocolate brown spots for both drugs following 30 minutes oven heating at 100 degrees C.

Results are in Table X of the Appendix.

5. Barbidex Tablets (Elliott - Marion)

Contents:

Amphetamine sulfate 15 mg.
Amobarbital 100 mg.

Procedure:

Two tablets were powdered in a mortar and pestle, transferred to a 50 ml. flask, and shaken with 20 ml. of ethanol

for 30 minutes. The mixture was filtered through a Whatman #2 filter disc employing suction, and then made up to volume in a 25 ml. volumetric flask. Similar standards were prepared.

Limits were established by spotting a series of concentrations. A set of plates with 12 micrograms of amphetamine and 80 micrograms of amobarbital were spotted and a second set with twice these amounts were also prepared. The plates were developed, dried and sprayed with acid-molybdate spray. The plates were charred at 120 degrees C. for 30 minutes. The spot size and R_f values were recorded and the plates scanned. Results are in Table XI of the Appendix.

6. Unitol Tablets (Horner)

Contents:

Amphetamine phosphate	5 mg.
Pentobarbital	32 mg.

Procedure:

Five tablets were reduced to a fine powder in a mortar and pestle, shaken with 15 ml. of 95 per cent ethanol for one-half hour, filtered with washing through a Whatman #2 disc, and made up to a volume of 20 ml. in a calibrated flask. Standards were prepared and two sets of plates run to establish parameters of assay cencentration. Ten (12.5 micrograms amphetamine and 80 micrograms pentobarbital) µl. of sample and standards were spotted on 10 plates, which were developed and subsequently sprayed with 50 per cent sulfuric acid for amphetamine and with acid-molybdate for pentobarbital. Results are in Table XII of the Appendix.

7. Ambar #2 Extentabs (A. H. Robins)

Contents:

Methamphetamine Hydrochloride	15 mg.
Phenobarbital	64.8 mg.

Procedure:

Five Ambar #2 Extentabs, were rinsed in a little water in a beaker to remove the color coating. After blotting with a paper towel, the tablets were crushed in a mortar and shaken mechanically for 30 minutes with 40 ml. of ethanol in a 125 ml. flask. The solution was filtered with washing through a Whatman #2 filter disc and made up to volume in a 50 ml. volumetric flask. Standards were prepared and the concentration range for assay determined. Sulfuric acid, 50 per cent, followed by 120 degrees centigrade heat for 30 minutes, was used to develop both drugs. The spots were brown on a pale grey background.

Results are in Appendix Table XIII.

8. Bitab #77 (Canada Pharmacal)

Contents:

Amphetamine sulfate	12 mg.
Phenobarbital	48 mg.

Procedure:

A mortar and pestle was used to powder 5 tablets which were then shaken with 45 ml. of ethanol for 30 minutes on a mechanical shaker. The excipients were removed by centrifugation for 15 minutes at 1500 revolutions per minute. In

this instance, centrifugation was more rapid than filtration due to the nature of the tablet excipients. The solution was decanted and made up to 60 ml. in a previously calibrated flask. A series of 10 plates were spotted with 20 micrograms amphetamine and 80 micrograms of phenobarbital contained in 20 microliters of sample and standards. Color-

ation was achieved with 50 per cent sulfuric acid followed by 30 minutes at 110 degrees C. The spots were grey-black on a pale grey background.

Results are in Appendix Table XIV.

9. Carbrital Kapseals (Parke, Davis and Co.)

Contents:

Carbromal	250 mg.
Pentobarbital sodium	100 mg.

Procedure:

To assay this product, 5 capsules were opened and the contents dissolved in exactly 50ml. of ethanol with the assistance of a mortar and pestle. This mixture did not require filtration. The upper and lower limits of the assay were set by running a series of several drug concentrations.

Carbromal stained easily with the furfural spray but the mercuric sulfate-dithizone reagent colored pentobarbital sodium with some difficulty. A new spray was developed using 2 per cent mercuric sulfate followed by 0.1 per cent sodium molybdate reagent and heat at 110 degrees C. for 30 minutes. Pentobarbital sodium appeared as a chocolate brown spot on a grey background. This was a more consistent spray for

pentobarbital than the mercuric sulfate-dithizone reagent. Results are in Appendix Table XV.

10. Edrisal Tablets (Smith, Kline and French)

Contents:

Acetylsalicylic acid (ASA)	160 mg.
Phenacetin	160 mg.
dl-Amphetamine	2.5 mg.

Procedure:

Six tablets were dissolved in 20 ml. of ethanol with the aid of a mortar and pestle. The material was shaken mechanically for 30 minutes of a 50 ml. flask and then filtered through activated charcoal on a Whatman #2 filter disc using suction. In this manner, the tablet excipients and most of the color was removed. The pad was rinsed with alcohol and the volume made up to 30 ml. in a calibrated flask.

A series of plates were run to establish concentration ranges and it was found that both the A.S.A. and phenacetin must be assayed separately from the dl-amphetamine due to the large concentration difference in the formulation. One set of plates were run to assay phenacetin and A.S.A. using 320 micrograms and another set were run using 10 micrograms of dl-amphetamine. Sufficient plates to allow 10 assays of each component were used.

After air and oven drying, the plates were sprayed with ferric chloride complex for phenacetin and A.S.A. and with 50 per cent sulfuric acid for dl-amphetamine.
Results are in Appendix Table XVI.

11. Tedral SA Tablets (Warner-Chilcott)

Contents:

Phenobarbital	25 mg.
Ephedrine HCl	48 mg.
Theophylline	180 mg.

Procedure:

Two tablets were finely powdered in a mortar with a pestle and were triturated and shaken with 25 ml. of warm ethanol. After mechanical shaking for one-half hour, the mixture was centrifuged at 1500 revolutions per minute for 15 minutes, decanted, and made up to 30 ml. in a calibrated flask. Centrifugation was again used to remove excess tablet excipent. Forty micrograms of phenobarbital, 80 micrograms of ephedrine, and 300 micrograms of theophylline of the standards and equivalent amounts of the sample were spotted on the plates.

Ephedrine was made visible with sulfuric acid and with Dragendorff's reagent. Theophylline was rendered visible with sulfuric acid and with the Iodine-hydrochloric acid spray. The phenobarbital was stained with sulfuric acid. Results are in Appendix Table XVII.

12. Daprisal Tablets (Smith, Kline and French)

Contents:

Amobarbital	32 mg.
Amphetamine Sulfate	5 mg.
Acetylsalicylic acid	160 mg.
Phenacetin	160 mg.

Procedure:

Five Daprisal tablets were finely powdered using a small mortar and pestle. The powder was mixed with 15 ml. of

ethanol and shaken mechanically for 30 minutes. A charcoal pad was prepared on a Whatman #2 filter disc and the solution filtered. This removed all insoluble material and most of the color. The pad was washed with a few ml. of alcohol and the volume brought up to 20 ml. in a previously calibrated flask. Standards were prepared in ethanol to contain equivalent amounts of the various drugs. During this procedure, it was found that the assay would have to be carried out in two parts. Amobarbital and amphetamine were assayed on one set of plates and acetylsalicylic acid and phenacetin on another set.

Amphetamine was detected by 50 per cent sulfuric acid, acetylsalicylic acid and phenacetin by ferric chloride complex and amobarbital by the mercuric sulfate-dithizone reagent. Results are in Appendix Table XVIII.

13. 217-Mep Tablets (C.E. Frosst)

Contents:

Acetylsalicylic acid (A.S.A.)	200 mg.
Phenacetin	150 mg.
Caffeine Citrate	30 mg.
Meprobamate	200 mg.

Procedure:

Two tablets were finely powdered and shaken for 30 minutes with 15 ml. of ethanol in a 25 ml. flask. The solution was filtered with rinsing through a Whatman #2 filter disc and made up to 20 ml. in a calibrated flask. Standards were made up and two sets of plates were prepared.

The assay had to be done in two parts due to the concentration difference between meprobamate and caffeine in the preparation. Meprobamate was detected with furfural, A.S.A. and phenacetin with ferric chloride complex and caffeine with Dragendorff's. Fufural also worked for caffeine, producing a yellow spot on a white background.

Results are in Appendix Table XIX.

14. Triple Sulfas Tablets (British Drug Houses)

Contents:

Sulfamethazine	167 mg.
Sulfamerazine	167 mg.
Sulfadiazine	167 mg.

Procedure:

The average weight per tablet was found to be 0.6105 G.

Ten tablets were powdered and a sample containing 10 micrograms of each sulfonamide was dissolved in 50 ml. of acetone. From 0.4 to 5 micrograms of each standard and an equivalent amount of each sample were spotted on a series of plates. The solvent suggested by A. Wehrli (48), containing 160 ml. chloroform, 40 ml. anhydrous methanol, and 2.5 ml. of distilled water, was used. After development, the plates were dried and sprayed with the Bratton-Marshall reagent spray. The spots are easily visible with a lower limit of about 0.5 microgram of sulfonamide. This assay is rapidly and accurately accomplished.

Results are in Appendix Table XX.

PART V

RESULTS

The results obtained for the standards, simulated mixtures, and the commercial preparations are listed in a series of tables, which include all pertinent information relative to the per cent recovery, and minimum and maximum assayable quantities.

Relationship Between Spot Area and Spot Weight

By preparing a series of known standards, it was possible to demonstrate that a quantitative relationship between spot area and concentration does exist and can be reproduced within certain parameters. This relationship was established for d-amphetamine sulphate in concentration ranges between 5 and 25 micrograms, for phenobarbital in concentrations between 40 and 120 micrograms and acetylsalicylic acid in concentration ranges between 50 and 250 micrograms. Following development and visualization of these drugs with the appropriate reagents, the spot areas showed quantitative correlation with drug content. The relevant data are given in Table I ,and plotted in Figures 10, 11, 12.

By comparing the integrated area measurement in "blips" with the actual area in square millimetres, it was calculated that each blip is equivalent to 12 square millimeters using the settings on the Densicord established by experience.

Table I

Data Showing Correlation Between Spot Weight And Spot Area

Drug	Amount applied micrograms	Area Sq. mm.
Amphetamine sulfate	5	60
	10	168
	15	204
	20	276
	25	348
Phenobarbital	40	372
	60	540
	80	660
	100	804
	120	936
Acetylsalicylic acid	50	240
	100	360
	150	528
	200	648
	250	780

Figure 10

Standard Curves for d-Amphetamine Sulfate
Illustating the Correlation between
Spot Weight and Spot Area

Response = 5

Filter = None

AREA AND WEIGHT APPLIED RELATIONSHIP

FOR AMPHETAMINE SULFATE

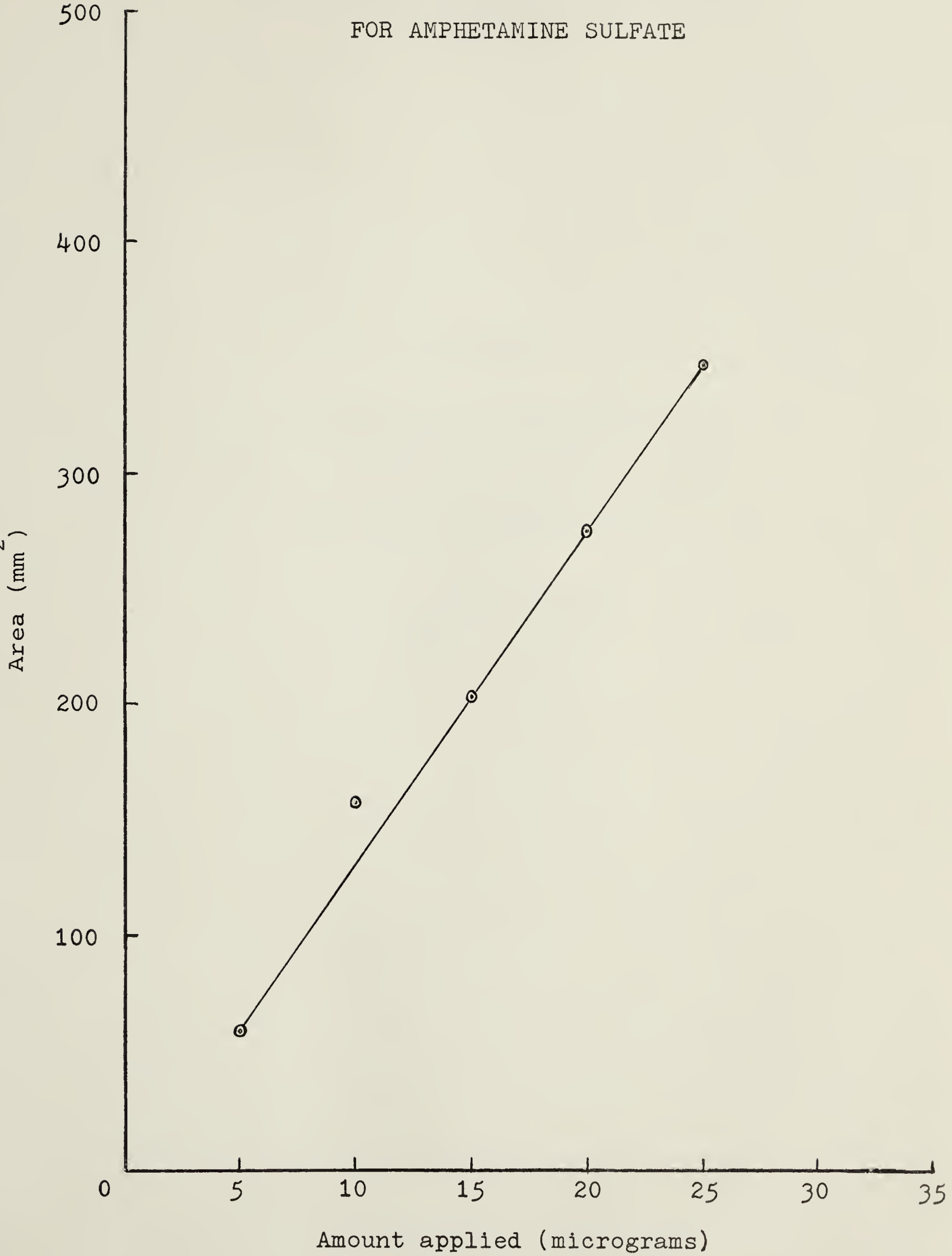


Figure 11

Standard Curve for Phenobarbital
Illustrating the Correlation between
Spot Weight and Spot Area

Response = 5
Filter = None

AREA AND WEIGHT APPLIED RELATIONSHIP

FOR PHENOBARBITAL

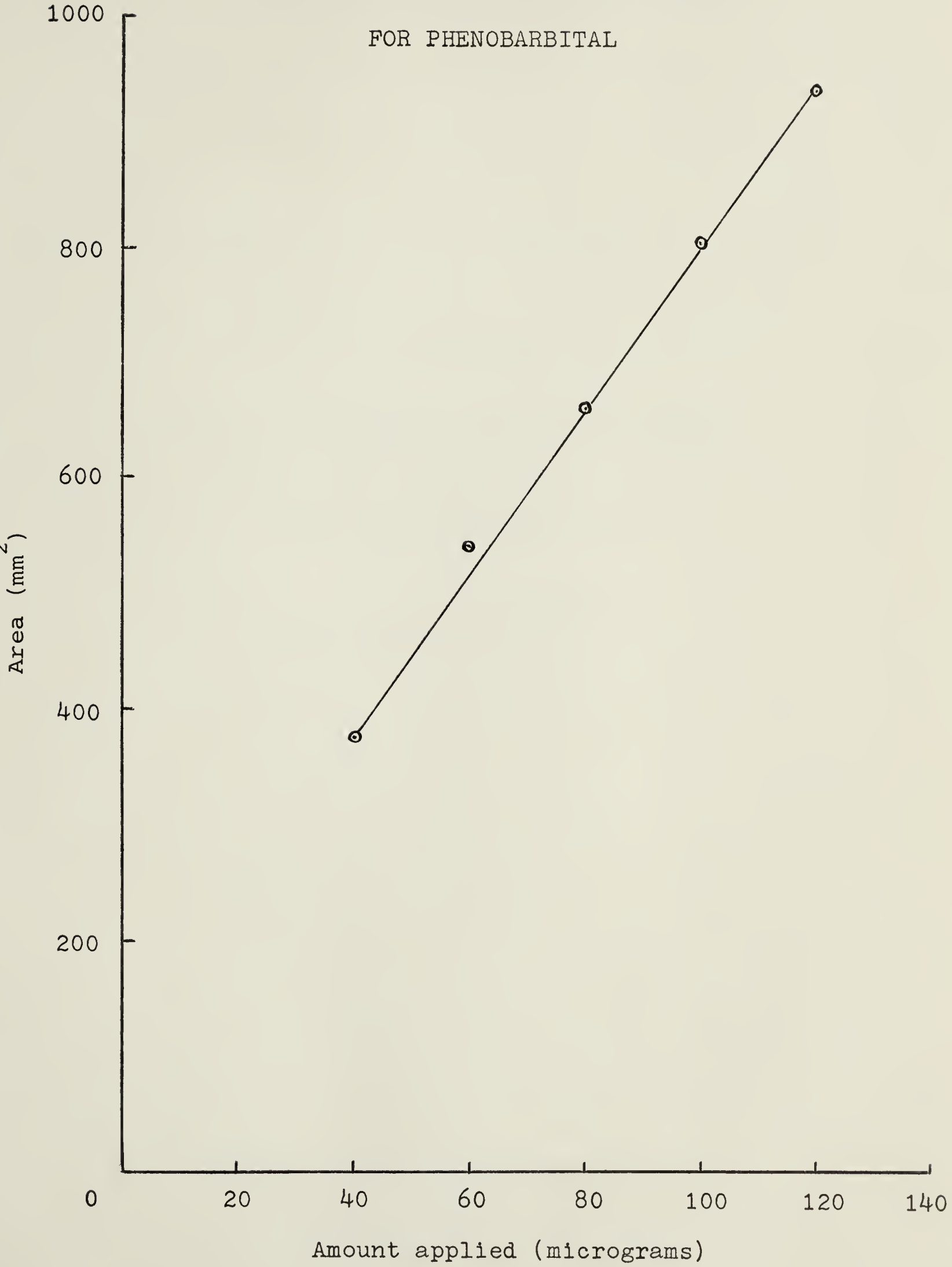


Figure 12

Standard Curve for Acetylsalicylic Acid

Illustrating the Correlation between

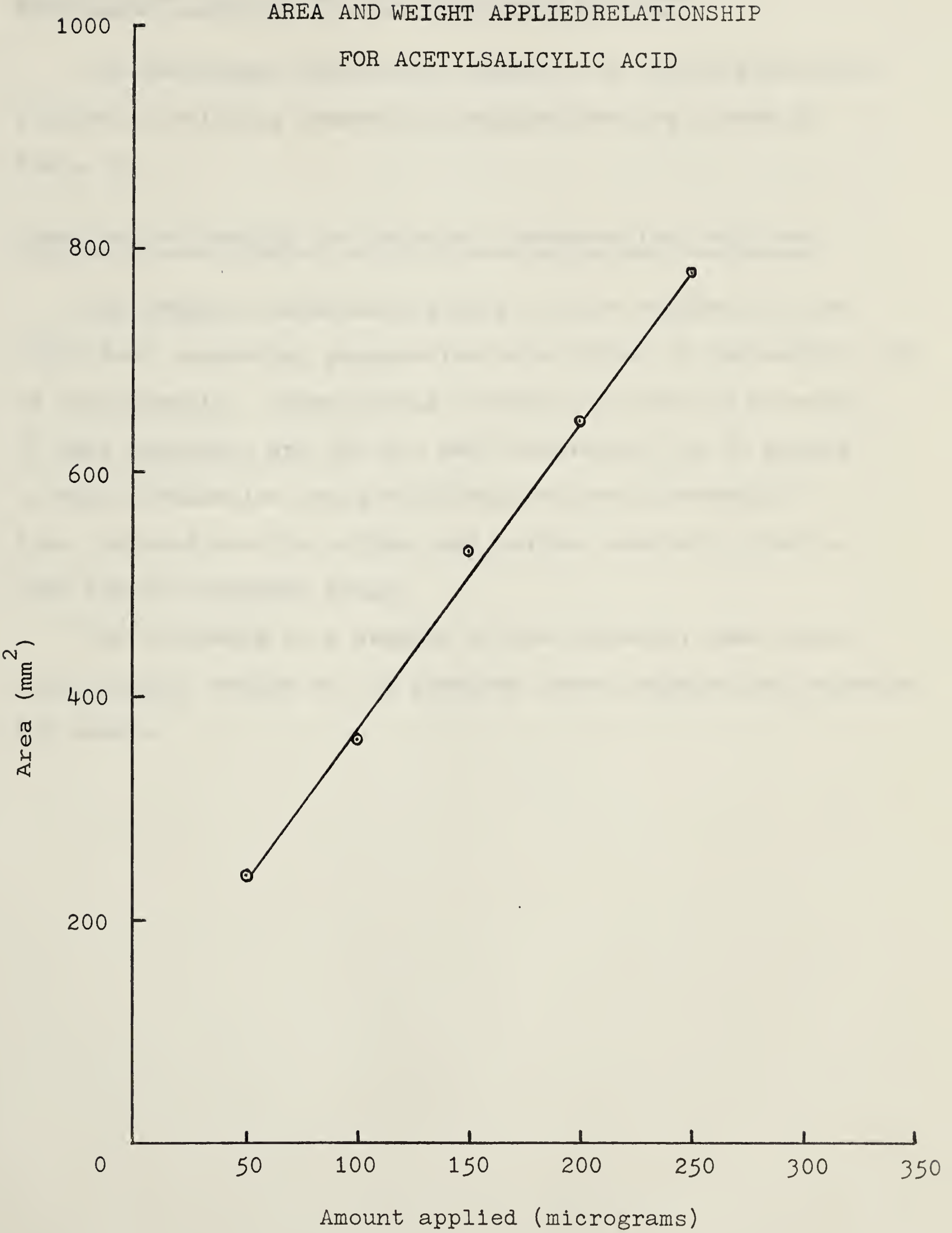
Spot Weight and Spot Area

Response = 5

Filter = Blue # 485

AREA AND WEIGHT APPLIED RELATIONSHIP

FOR ACETYLSALICYLIC ACID



Quantitative Results for Simulated Drug Mixtures

The percentage recoveries obtained for the drug mixtures simulating existing commercial preparations are listed in Table II.

Quantitative Results for Selected Pharmaceutical Mixtures

The complete experimental data for the analysis of the individual commercial preparations are listed in Tables VII - XX of the Appendix. These Tables include the labelled strength of each component and the per cent recoveries for 10 assays on each preparation using the described assay procedure. Also included are the minimum and maximum assayable quantities for the relevant drugs.

The following is a summary of the contents, mean recoveries, and Rf values of the fourteen trade preparations selected for assay.

Table II

Quantitative Results for Simulated Drug Mixtures

Drug	Amount per/tablet (milligrams)	Mean Per cent Recovery	R_f Values
Simulated Bitab # 77			
d-Amphetamine sulfate	12.0	96.4	0.44
Phenobarbital	48.0	97.0	0.62
Simulated Bamadex Tablets			
d-Amphetamine sulfate	5.0	99.7	0.21
Meprobamate	400.0	98.3	0.51
Simulated Portion of Daprisal Tablets			
Amobarbital	32.0	95.4	0.79
Phenacetin	160.0	98.7	0.48

Table III

Quantitative Results for Selected Pharmaceuticals

Trade Preparation	Labelled Amount/tablet (milligrams)	Mean Per cent Recovery	R_f Values
Bamadex (tablets)			
d-Amphetamine sulfate	5.0	99.4 ± 3.4	0.22
Meprobamate	400.0	98.0 ± 3.3	0.49
Eskatrol (tablets)			
d-Amphetamine sulfate	5.0	94.5 ± 2.7	0.21
Prochlorperazine maleate	2.5	92.6 ± 3.5	0.38
Dexamyl (tablets)			
d-Amphetamine sulfate	5.0	94.6 ± 4.8	0.29
Amobarbital	32.0	93.7 ± 2.6	0.85
Butadex (tablets)			
d-Amphetamine sulfate	5.0	97.1 ± 5.2	0.27
Butabarbital sodium	32.0	94.1 ± 1.7	0.55
Barbidex (tablets)			
d-Amphetamine sulfate	15.0	95.8 ± 5.6	0.31
Amobarbital	100.0	95.9 ± 2.2	0.86
Unitol (tablets)			
d-Amphetamine phosphate	5.0	96.3 ± 3.9	0.44
Pentobarbital	32.0	94.2 ± 2.6	0.73
Ambar # 2 (tablets)			
Methamphetamine HCl	15.0	94.0 ± 2.9	0.20
Phenobarbital	64.8	95.2 ± 5.0	0.85
Bitab # 77 (tablets)			
d-Amphetamine sulfate	12.0	97.9 ± 4.9	0.46
Phenobarbital	48.0	96.2 ± 3.9	0.60

Trade Preparation	Labelled Amount/tablet (milligrams)	Mean Per cent Recovery	R_f Values
Carbrital (capsules)			
Carbromal	250.0	96.4 ± 4.3	0.73
Pentobarbital	100.0	95.6 ± 3.4	0.59
Edrisal (tablets)			
Acetylsalicylic acid	160.0	98.9 ± 5.8	0.00
Phenacetin	160.0	98.5 ± 6.5	0.47
dl-Amphetamine	2.5	99.4 ± 3.8	0.18
Tedral SA (tablets)			
Phenobarbital	25.0	97.3 ± 2.7	0.40
Ephedrine HCl	48.0	91.8 ± 3.3	0.18
Theophylline	180.0	no recovery	0.00
Daprisal (tablets)			
Amobarbital	32.0	99.1 ± 3.5	0.72
d-Amphetamine sulfate	5.0	100.6 ± 2.9	0.23
Acetylsalicylic acid	160.0	96.5 ± 3.3	0.00
Phenacetin	160.0	96.4 ± 3.7	0.51
217-Mep (tablets)			
Acetylsalicylic acid	200.0	93.8 ± 3.0	0.00
Phenacetin	150.0	93.8 ± 3.4	0.63
Caffeine citrate	30.0	96.6 ± 2.5	0.53
Meprobamate	200.0	96.9 ± 3.5	0.45
Triple Sulfas (tablets)			
Sulfamethazine	167.0	96.6 ± 4.9	0.75
Sulfadiazine	167.0	94.5 ± 2.7	0.63
Sulfamerazine	167.0	96.3 ± 3.8	0.71

PART VI

DISCUSSION

Several methods are available for quantitatively determining the composition of a mixture resolved by thin-layer chromatography. These methods are essentially micro-analytical since the quantity of drug or drugs applied to the chromatogram is very small.

Although the most obvious, gravimetric methods have several disadvantages. The drug can be so strongly adsorbed that it may not be completely eluted from the silica and the adsorbent itself may contain extractable impurities which introduce errors into a gravimetric method. The main disadvantage, however, is that the total amount of drug recoverable from the plate may only be a few milligrams, an amount small enough to require special techniques.

Compounds which absorb in the ultra-violet and visible regions can be determined by spectrophotometric methods following their elution from the adsorbent. Kirchner and others (73) determined the components of citrus fruits by this method and, in a series of determinations, found the average error to be 2.8 per cent. Using the same technique, Stanley and Vannier (74) determined the coumarin derivatives in lemon oil and Schlemmer and Link (75) estimated the concentration of reserpine and rescinnamine in samples of Rauwolfia. In the latter study, the average deviation of five determinations for each compound was 3.5 per cent.

The two main sources of error in the spectrophotometric method of estimation are the elution of impurities from the

adsorbent and the incomplete recovery of the compound from the adsorbent. For example, attempts to elute amphetamine sulfate from the silica gel gave recoveries of almost 400 per cent when assayed spectrophotometrically (76). This simultaneous estimation of ultra-violet absorbing impurities may be eliminated by increasing the wavelength at which the drug is estimated. This is done by subjecting the compound to a colorimetric reaction and subsequently estimating the intensity of the color produced as a function of concentration. Initial studies were conducted along these lines but it was found that many of the relevant color-producing reagents lacked sufficient sensitivity in the range of concentration practicable for thin-layer chromatographic work.

Since much recent work has stressed the relationship of spot size to drug content because of the inherent simplicity of the technique, it was decided to devise a method of optical-densitometric estimation because a chromogenic process, unaffected by impurities in the adsorbent, eliminates the need for elution or extraction of the compound (77 - 79).

Techniques based on area measurement are simple to operate and have an accuracy comparable to or better than gravimetric or spectrophotometric techniques. Furthermore, in using a photo-electric device, the process of measurement is rapid and convenient. Such an instrument allows analytical procedures to become automated when their reliability has been established. These procedures can then be carried out

by semi-skilled operators. In employing specific reagents to render the drug or drugs visible on the chromatoplate, the technique nullifies the effect of any impurities present in the adsorbent and ensures that only the drug is estimated.

Hefendahl (80) established that the size and optical density of a spot are proportional to the weight of material it contains. Several other workers have verified this conclusion and established that the relationship appears to be valid for quantities ranging from 50 to 100 micrograms. Morrison and Chatten (81) established that drug mixtures containing antihistamines could be estimated from spot size when the concentration of the applied drug was in the region of 25 to 50 micrograms. The error calculated in their procedure was approximately 3 per cent.

From the results obtained in this study, it is apparent that the technique of using specific spray reagents, followed by densitometric estimation, is sufficiently accurate for routine pharmaceutical analysis and can be applied to many pharmaceutical formulations and dosage forms. The average error was found to be about 5 per cent. The commercial products in capsule form required less manipulation in the assay because they seldom contain excipients which must be removed either by filtration or centrifugation. The total assay time, including plate development, for a two-component tablet was found to be about five and one-half hours. A two-component capsule could be assayed in about four and one-half hours.

The actual analysis time, however, is determined by the scanning speed of the instrument which is set at one inch per minute. Since only one-half or less of an eight inch plate will be scanned at any one time, quantitative results may be obtained in about five minutes. It should be observed that while the plates are being developed in the covered tanks, the operator is free to carry out other duties.

The Photovolt Densicord densitometer is designed to produce quantitative results by measuring photo-electrically the difference in intensity between the visible spot and the background of the plate, i.e., the optical density of the background is set at zero and the intensity of the spot is measured relative to this. If the plate background is colored, its photo-intensity will be high and the contrast with the spot will be diminished. This results in decreased pen excursion traced by the recorder so that the areas produced by the recorded densities may not be particularly reliable. For this reason it is essential to use spray reagents which produce specific stains for individual drugs and do not color the background to any significant extent. If a satisfactory color cannot be developed, it is doubtful whether the method could be used for quantitative work.

Several experimental factors are critical in obtaining consistent and accurate results. Failure to recognize these may result in significant deviations in the correlation between spot density and spot weight. The applied and developed

spot size should be as uniform as possible to ensure reproducible results. The area of application for the applied spot should be between six and eight millimeters, since the area of the spot may increase during development from 25 to 100 per cent (Appendix Table XXI). If the spots are not uniformly applied, solutions containing equal quantities of drug may occupy different areas and this difference with its consequent error will be registered by the densitometer. The tailing of spots caused by overloading the plate should be avoided for similar reasons. Irregularity in spot densi. will give irregular results since the densitometer records only what the photo-electric cell " sees ". It is also necessary to spray the plates as evenly as possible to ensure even coloration of the spots. In practice, a weave pattern (cross-check) was found to be the most useful, with the sprayer held far enough from the plate to avoid damaging the layer of silica.

It is desirable to have the layer of silica gel spread in as uniform a layer as possible. Some variation may inadvertently occur but compensation for slight gradients in thickness can be made by scanning the plate at right angles to the gradient. Variations in layer thickness cause alter - ations in the light reflected by the background and so produce inconsistent results since it is this difference between background and spot intensity which the instrument measures. For this reason, it is important to clean the

underside of the plate before scanning, to remove any material which might absorb or reflect light and influence the density observed by the photocell.

For thin-layer chromatographic analysis using densitometry, it is essential that the drugs being assayed separate sharply and distinctly from each other. If the spots overlap even slightly, any technique utilizing area measurements is liable to error. It is possible to counter-act the effects of overlap to some extent by spraying for each drug with a stain which will color only that drug and not any other. Two or more plates must be used for each component and mixtures with several ingredients lengthen the procedure considerably, hence this method is not satisfactory. In several of the assay procedures, the phenomena known as " edge effect " was observed(82). This is a physical occurance in which the spots at either edge of the plate have a higher migration rate than those spots in the center. It is caused mainly be more rapid solvent evaporation at the edge of the plate and this causes a slight acceleration in drug migration rate. This effect is virtually eliminated by lining the chromatographic developing tanks with filter paper and allowing sufficient time for equilibration. The outside spot on a plate may occasionally show this phenomena even with such tank lining but since several spots are employed on a plate it may be disregarded.

The minimum detectable quantity observed for a drug on a thin-layer plate is largely a function of the specific spray reagent used to produce a distinctive color but these chemical stains vary in their relative sensitivities of detection. With the exception of phenobarbital, the barbiturates did not appear distinctly after charring with sulfuric acid. When a 2 per cent mercuric sulfate spray was used followed by 0.1 per cent sodium molybdate in concentrated sulfuric acid, both amobarbital and butabarbital appeared as distinct brown spots on a grey background. As a result, the minimum assayable quantity for these barbiturates using the acid-molybdate spray was 40 micrograms as opposed to 50 micrograms in the sulfuric acid spray. Cobalt nitrate reagent required 100 micrograms of amobarbital to produce a spot suitable for assay.

The tetra-azotized benzidine spray produced the most diffuse spots with hazy and indistinct edges and for this reason was seldom used. The furfural reagent could detect meprobamate at weights of less than 80 micrograms but required more than 100 micrograms of carbromal to produce consistent spots.

The selection of a solvent system suitable for separation of a specific drug must still be approached empirically. The solvent systems selected in this study were chosen on the basis of trial and error. In addition, solvent systems reported in the literature as successful in separating certain

mixtures, inexplicably failed to separate similar mixtures when used in this study. Recent workers (83,84) have examined the problem of solvent selection from a physico-chemical standpoint but there does not appear to be a rational basis as yet for the selection of satisfactory solvents. Determination of a solvent can be qualitatively accomplished by finding the R_f values for each component of a mixture on a slica gel coated glass microscope slide. A 250 ml. beaker covered with aluminum foil makes a satisfactory developing chamber and development occurs in about 20 minutes. In this way, a large number of solvents can be examined very rapidly.

It may be necessary to vary the amount of solution applied to a plate in such a way that the amount of drug being estimated contains the minimum detectable quantity. For example, Bamadex tablets have a ratio of amphetamine to meprobamate of one to eighty and the amounts of solution applied to the plate during the assay of this preparation must be varied accordingly. This usually entails a separate assay for each component on separate plates and does not raise any particular experimental difficulties.

Commercial preparations containing drugs which have very low solubilities in most chemical solvents raised some problems. Due to the very low solubility of prochlorperazine maleate in extracting solvents, the agitation time during extraction was increased from 30 to 60 minutes when assaying Eskatrol. Solubility was also a major factor in the assay of

Tedral S A tablets when it was found that theophylline had such a low solubility and was so slowly soluble that quantitative estimation by this method was impossible. The procedure was further complicated by the variability of the stain used to color theophylline. Recovery was less than 60 per cent.

Thin-layers of cellulose were used initially in an attempt to avoid the problem of interfering contaminants present in the silica gel and thus allow the use of elution techniques. However, cellulose suffers from several disadvantages, the main one being a significant lack of absorptive power. It was found impractical to apply a large volume of liquid to thin layers of cellulose because the resulting spot size was too large and too diffuse to allow quantitative measurement. A second disadvantage is the narrow range of spray reagents which can be used for detection. Common sprays such as sulfuric acid or ceric sulfate destroy the cellulose background by charring.

The purpose of including the Trisulfapyramidines tablets U. S. P., in this study was to demonstrate the application of the densitometric technique to systems where distinct stains exist or can easily be found. Using the method reported by Wehrli (48) for the qualitative separation of these sulfonamides, it was found that a quantitative evaluation could be readily carried out by the described method.

It is suggested that if a sensitive spray reagent exists and the various experimental conditions are observed, the densitometric method of assay should be routinely applicable in pharmaceutical analysis to yield quantitative results.

1. A thin-layer chromatographic technique, using silica gel G as the adsorbent, has been devised for the separation of fourteen selected pharmaceuticals which included amphetamines, certain barbiturates, and several related cpmpounds.
2. It was demonstrated that a linear relationship exists between spot area and spot content. Quantitative evaluation was achieved without elution from the adsorbent by employing a photo-electric densitometer coupled to an electronic integrator which computed the spot areas. The experimental error was found to be approximately 5 per cent.
3. The technique was shown to be quantitative when the drugs were applied within certain concentrations. These concentrations varied with individual drugs but were in the range of 25 to 100 micrograms generally.
4. The applicability of the method was illustrated by obtaining quantitative recoveries from single drug standards and simulated drug mixtures as well as the selected pharmaceuticals. Results were obtained more rapidly and with greater convenience than by planimetry or visual area measurements.
5. Several selective spray reagents are described which proved satisfactory for the drugs studied.
6. Several experimental factors which influence quantitative recovery are discussed.

7. Dioxane-benzene-25 per cent ammonia (40:50:10 v/v) was found satisfactory for resolving all the selected pharmaceuticals into their respective components.

8. The technique could be applied routinely in micro-analysis and has specific application to pharmaceuticals.

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APPENDIX

Table IV

Quantitative Results for Simulated Bitab #77

Composition:

d-Amphetamine SO_4	12 mg.
Phenobarbital	48 mg.

Spotting Solvent: Ethanol 95 per cent

Developing Solvent: Dioxane:Benzene: 25 per cent Ammonia (40:50:10)

Quantity applied:
10 + 20 µg. Amphetamine
48 + 96 µg. Phenobarbital

Quantity recovered: (per cent)

		Amphetamine	Phenobarbital
Assay number	1.	95.8	100.0
	2.	97.1	95.4
	3.	93.2	98.0
	4.	96.0	93.6
	5.	100.0	97.8
Mean		96.4	97.0

	Amphetamine	Phenobarbital
Minimum detectable quantity:	5 µg.	40 µg.
Minimum assayable quantity:	10 µg.	50 µg.
Maximum assayable quantity:	200 µg.	300 µg.

Instrument Settings:

a. Response: 5

b. Chart Speed: 2 inches per minute

c. Filter: none

Total Analysis Time: 3 hours for 5 assays

Table V

Quantitative Results for Simulated Bamadex Tablets

Composition:

d-Amphetamine SO_4	5 mg.
Meprobamate	400 mg.

Spotting Solvent: Ethanol 95 per cent

Developing Solvent: Dioxane:Benzene: 25 per cent Ammonia (40:50:10)

Quantity applied:

Amphetamine	10 µg.
Meprobamate	800 µg.

Quantity recovered: (per cent)

	Amphetamine	Meprobamate
Assay number 1.	100.0	98.0
2.	100.0	98.6
3.	95.2	98.4
4.	106.3	101.0
5.	97.1	95.3
Mean	99.7	98.3

	Amphetamine	Meprobamate
Minimum detectable quantity:	5 µg.	80 µg.
Minimum assayable quantity:	10 µg.	80 µg.
Maximum assayable quantity:	200 µg.	1600 µg.

Instrument Settings:

a. Response 5

b. Chart Speed: 2 inches per minute

c. Filter: none

Total Analysis Time: 3 hours for 5 determinations

Table VI

Quantitative Results for Simulation of Part of Daprisal Tablets

Composition:

Amobarbital	32 mg.
Phenacetin	160 mg.

Spotting Solvent: Ethanol 95 per cent

Developing Solvent: Dioxane:Benzene: 25 per cent Ammonia (40:50:10)

Quantity applied: Amobarbital 40 µg. Amobarbital 80 µg.
Phenacetin 200 µg. Phenacetin 400 µg.

Quantity recovered: (per cent)

		Amobarbital	Phenacetin
Assay number	1.	94.6	100.0
	2.	95.0	93.2
	3.	94.7	103.3
	4.	97.2	98.1
Mean		95.4	98.7

	Amobarbital	Phenacetin
Minimum detectable quantity:	40 µg.	50 µg.
Minimum assayable quantity:	50 µg.	50 µg.
Maximum assayable quantity:	300 µg.	300 µg.

Instrument Settings:

a. Response: 5

b. Chart Speed: 2 inches per minute

c. Filter: #595

Total Analysis Time: 3 hours for 5 determinations

Table VII

Quantitative Results for Bamadex Tablets

Composition:

d-Amphetamine Sulphate 5 mg.
Meprobamate 400 mg.

Spotting Solvent: Ethanol 95 per cent

Developing Solvent: Dioxane:Benzene: 25 per cent Ammonia (40:50:10)

Quantity applied: Amphetamine 10 µg.
Meprobamate 800 µg.

Quantity recovered: (per cent)		Amphetamine Sulphate	Meprobamate
Assay number	1.	100.0	95.6
	2.	103.0	95.7
	3.	102.3	98.0
	4.	103.5	100.6
	5.	103.1	96.5
	6.	97.6	95.1
	7.	95.5	105.4
	8.	95.7	97.7
	9.	97.8	93.9
	10.	95.1	99.6
Mean		99.4	98.0
Standard Deviation		± 3.4	± 3.3

	Amphetamine	Meprobamate
Minimum detectable quantity:	10 µg.	80 µg.
Minimum assayable quantity:	10 µg.	80 µg.
Maximum assayable quantity:	20 µg.	1600 µg.

Instrument Settings:

a. Response: 5

b. Chart Speed 2 inches per minute

c. Filter: none

Total Analysis Time: 5 1/2 hours for 10 determinations

Table VIII

Quantitative Results for Eskatrol Tablets

Composition:

d-Amphetamine Sulphate 5 mg.
Prochlorperazine Maleate 2.5 mg.

Spotting Solvent: Ethanol 95 per cent

Developing Solvent: Dioxane:Benzene: 25 per cent Ammonia (40:50:10)

Quantity applied: Amphetamine 20 µg.
Prochloperazine 10 µg.

Quantity recovered: (per cent)		Amphetamine	Prochlorperazine
Assay number	1.	93.8	87.5
	2.	94.4	88.5
	3.	95.6	90.0
	4.	91.3	94.1
	5.	100.0	94.6
	6.	95.6	91.7
	7.	95.2	93.8
	8.	92.6	100.0
	9.	96.3	95.0
	10.	90.0	90.5
Mean		94.5	92.6
Standard Deviation		± 2.7	± 3.5

	Amphetamine	Prochlorperazine
Minimum detectable quantity:	5 µg.	5 µg.
Minimum assayable quantity:	10 µg.	10 µg.
Maximum assayable quantity:	200 µg.	150 µg.

Instrument Settings:

a. Response: 5

b. Chart Speed: 2 inches per minute

c. Filter: none

Total Analysis Time: 6 hours for 10 determinations

Table IX

Quantitative Results for Dexamyl Tablets

Composition:

d-Amphetamine Sulphate	5 mg.
Amobarbital	32 mg.

Spotting Solvent: Ethanol 95 per cent

Developing Solvent: Dioxane:Benzene: 25 per cent Ammonia (40:50:10)

Quantity applied:

Amphetamine	12.5 µg.
Amobarbital	80 µg.

Quantity recovered: (per cent)

	Amphetamine	Amobarbital
Assay number 1.	94.7	92.8
2.	92.3	96.2
3.	93.8	94.3
4.	90.0	91.4
5.	88.9	100.0
6.	95.4	91.9
7.	90.5	91.7
8.	95.0	93.3
9.	100.0	94.1
10.	105.6	90.9
Mean	94.6	93.7
Standard Deviation	± 4.8	± 2.6

	Amphetamine	Amobarbital
Minimum detectable quantity:	5 µg.	40 µg.
Minimum assayable quantity:	10 µg.	50 µg.
Maximum assayable quantity:	200 µg.	300 µg.

Instrument Settings:

a. Response: 5

b. Chart Speed: 2 inches per minute

c. Filter: none

Total Analysis Time: 5 1/2 hours for 10 determinations

Table X

Quantitative Results for Butadex Tablets

Composition:

d-Amphetamine Sulphate 5 mg.
Butabarbital Sodium 32 mg.

Spotting Solvent: Ethanol 95 per cent

Developing Solvent: Dioxane:Benzene: 25 per cent Ammonia (40:50:10)

Quantity applied: Amphetamine 12.5 µg.
Butabarbital 80 µg.

Quantity recovered: (per cent)

		Amphetamine	Butabarb
Assay number	1.	93.3	95.2
	2.	100.0	92.4
	3.	100.0	95.2
	4.	100.0	95.6
	5.	92.9	96.7
	6.	90.0	95.0
	7.	100.0	92.6
	8.	93.3	94.1
	9.	92.9	93.3
	10.	108.3	91.1
Mean		97.1	94.1
Standard Deviation		± 5.2	± 1.7

	Amphetamine	Butabarb
Minimum detectable quantity:	5 µg.	40 µg
Minimum assayable quantity:	10 µg.	50 µg.
Maximum assayable quantity:	200 µg.	300 µg.

Instrument Settings:

a. Response: 5

b. Chart Speed: 2 inches per minute

c. Filter: none

Total Analysis Time: 6 hours for 10 determinations

Table XI

Quantitative Results for Barbidex Tablets

Composition:

d-Amphetamine Sulphate	15 mg.
Amobarbital	100 mg.

Spotting Solvent: Ethanol 95 per cent

Developing Solvent: Dioxane:Benzene: 25 per cent Ammonia (40:50:10)

Quantity applied:

Amphetamine	12 µg.
Amobarbital	80 µg.

Quantity recovered: (per cent)

	Amphetamine	Amobarbital
Assay number 1.	91.7	93.3
2.	91.4	94.4
3.	97.1	98.5
4.	91.7	98.1
5.	96.4	91.2
6.	96.0	98.5
7.	95.4	96.2
8.	103.6	95.8
9.	92.3	96.1
10.	103.2	96.6
Mean	95.8	95.9
Standard Deviation	± 5.6	± 2.2

	Amphetamine	Amobarbital
Minimum detectable quantity:	5 µg.	40 µg.
Minimum assayable quantity:	10 µg.	50 µg.
Maximum assayable quantity:	200 µg.	300 µg.

Instrument Settings:

a. Response: 5

b. Chart Speed: 2 inches per minute

c. Filter: none

Total Analysis Time: 5 1/2 hours for 10 determinations

Table XII

Quantitative Results for Unitol Tablets

Composition:	d-Amphetamine Phosphate 5 mg. Pentobarbital 32 mg.		
Spotting Solvent:	Ethanol 95 per cent		
Developing Solvent:	Dioxane:Benzene: 25 per cent Ammonia (40:50:10)		
Quantity applied:	Amphetamine 12.5 µg. Pentobarbital 80 µg.	Amphetamine 25 µg. Pentobarbital 160 µg.	

Quantity recovered: (per cent)

		Amphetamine	Pentobarbital
Assay number	1.	93.7	95.7
	2.	100.6	95.8
	3.	94.7	92.1
	4.	92.3	100.0
	5.	90.7	94.1
	6.	97.9	94.7
	7.	94.6	94.1
	8.	94.7	90.0
	9.	102.4	92.3
	10.	102.1	93.1
Mean		96.3	94.2
Standard Deviation		± 3.9	± 2.6

	Amphetamine	Pentobarbital
Minimum detectable quantity:	5 µg.	40 µg.
Minimum assayable quantity:	10 µg.	50 µg.
Maximum assayable quantity:	200 µg.	300 µg.

Instrument Settings:

a. Response: 5

b. Chart Speed: 2 inches per minute

c. Filter none

Total Analysis Time: 6 hours for 10 determinations

Table XIII

Quantitative Results for Ambar #2 Extentabs

Composition:

Methamphetamine Hydrochloride 15 mg.
Phenobarbital 64.8 mg.

Spotting Solvent: Ethanol 95 per cent

Developing Solvent: Dioxane:Benzene: 25 per cent Ammonia (40:50:10)

Quantity applied: Methamphetamine 15 µg. Phenobarbital 62.5 µg. Methamphetamine 30 µg. Phenobarbital 130 µg.

Quantity recovered: (per cent)

		Methamphetamine	Phenobarbital
Assay number	1.	94.9	95.1
	2.	100.0	98.6
	3.	93.1	99.3
	4.	89.5	94.6
	5.	91.9	95.3
	6.	94.4	98.1
	7.	97.6	91.2
	8.	91.2	95.0
	9.	94.3	92.0
	10.	92.9	93.7
Mean		94.0	95.2
Standard Deviation		± 2.9	± 5.0

	Methamphetamine	Phenobarbital
Minimum detectable quantity:	5 µg.	40 µg.
Minimum assayable quantity:	10 µg.	50 µg.
Maximum assayable quantity	200 µg.	350 µg.

Instrument Settings:

a. Response: 5

b. Chart Speed: 2 inches per minute

c. Filter: none

Total Analysis Time: 5 1/2 hours for 10 determinations

Table XIV

Quantitative Results for Bitab #77 Tablets

Composition:

d-Amphetamine Sulphate	12 mg.
Phenobarbital	48 mg.

Spotting Solvent: Ethanol 95 per cent

Developing Solvent: Dioxane:Benzene: 25 per cent Ammonia (40:50:10)

Quantity applied:

Amphetamine	20 µg.
Phenobarbital	80 µg.

Quantity recovered: (per cent)

	Amphetamine	Phenobarbital
Assay number 1.	100.0	96.0
2.	96.4	94.3
3.	95.8	97.1
4.	97.9	96.5
5.	102.9	92.9
6.	100.0	106.5
7.	91.7	90.7
8.	95.6	94.3
9.	107.9	96.1
10.	90.5	97.9
Mean	97.9	96.2
Standard Deviation	± 4.9	± 3.9

	Amphetamine	Phenobarbital
Minimum detectable quantity:	5 µg.	40 µg.
Minimum assayable quantity:	10 µg.	50 µg.
Maximum assayable quantity:	200 µg.	350 µg.

Instrument Settings:

a. Response: 5

b. Chart Speed: 2 inches per minute

c. Filter: none

Total Analysis Time: 5 1/2 hours for 10 determinations

Table XV

Quantitative Results for Carbrital Capsules

Composition:

Carbromal	250 mg.
Pentobarbital Sodium	100 mg.

Spotting Solvent: Ethanol 95 per cent

Developing Solvent: Dioxane:Benzene: 25 per cent Ammonia (40:50:10)

Quantity applied:

Carbromal	250.0 µg.
Pentobarbital	100.0 µg.

Quantity recovered: (per cent)

	Carbromal	Pentobarbital
Assay number 1.	102.9	90.5
2.	95.1	100.0
3.	95.7	98.1
4.	90.6	90.9
5.	92.9	97.0
6.	96.4	100.0
7.	94.5	94.3
8.	105.3	91.4
9.	93.4	97.1
10.	96.7	97.1
Mean	96.4	95.6
Standard Deviation	± 4.3	± 3.4

	Carbromal	Pentobarbital
Minimum detectable quantity:	85 µg.	40 µg.
Minimum assayable quantity:	100 µg.	50 µg.
Maximum assayable quantity:	300 µg.	300 µg.

Instrument Settings:

a. Response: 5

b. Chart Speed: 2 inches per minute

c. Filter: none

Total Analysis Time: 4 1/2 hours for 10 determinations

Table XVI

Quantitative Results for Edrisal Tablets

Composition:

Acetylsalicylic Acid	160 mgm.
Phenacetin	160 mgm.
Benzedrine	2.5 mgm.

Spotting Solvent: Ethanol 95 per cent

Developing Solvent: Dioxane:Benzene: 25 per cent Ammonia (40:50:10)

Quantity applied:

Benzedrine	10 µg.
ASA + Phenacetin	320 µg.

Quantity recovered (per cent)

	A.S.A.	Phenacetin	Benzedrine
Assay number 1.	94.4	100.0	100.0
2.	95.2	100.0	106.3
3.	90.5	100.0	102.3
4.	100.5	109.1	102.5
5.	92.3	92.3	86.9
6.	100.0	107.7	102.9
7.	105.0	91.7	97.2
8.	103.7	100.0	97.1
9.	100.0	92.3	95.0
10.	108.3	91.7	93.8
Mean	98.9	98.5	99.4
Standard Deviation	± 5.8	± 6.5	± 3.8

	Phenacetin	Benzedrine	A.S.A.
Minimum detectable quantity:	50 µg.	10 µg.	50 µg.
Minimum assayable quantity	50 µg.	10 µg.	50 µg.
Maximum assayable quantity:	350 µg.	350 µg.	350 µg.

Instrument Settings:

a. Response: 5

b. Chart Speed: 2 inches per minute

c. Filter: For Phenacetin and A.S.A. - #485 Blue

Total Analysis Time: 6 hours for 10 determinations

Table XVII

Quantitative Results for Tedral SA Tablets

Composition:

Phenobarbital	25 mg.
Ephedrine	48 mg.
Theophylline	180 mg.

Spotting Solvent: Ethanol 95 per cent

Developing Solvent: Dioxane:Benzene: 25 per cent Ammonia (40:50:10)

Quantity recovered: (per cent)		Phenobarbital	Ephedrine	Theophylline
Assay number	1.	96.6	89.7	spots not
	2.	98.7	100.0	assayable
	3.	102.3	89.5	
	4.	100.0	87.5	
	5.	98.9	90.9	
	6.	96.4	92.0	
	7.	92.0	92.2	
	8.	95.7	89.4	
	9.	95.1	95.0	
Mean		97.3	91.8	
Standard Deviation		± 2.7	± 3.3	

Minimum detectable quantity: Ephedrine 10 µg., Phenobarb 40µg.

Minimum assayable quantity: Ephedrine 10 µg., Phenobarb 50µg.

Maximum assayable quantity: Ephedrine 200 µg., Phenobarb 300µg.

Instrument Settings:

a. Response: 5

b. Chart Speed: 2 inches per minute

c. Filter: none

Total Analysis Time: 5 1/2 hours for 9 determinations

Quantity applied:

Phenobarbital	42 µg.
Ephedrine	80 µg.
Theophylline	300 µg.

Table XVIII

Quantitative Results for Daprisal Tablets

Composition:

Amobarbital	32 mg.
d-Amphetamine	5 mg.
Acetylsalicylic acid	160 mg.
Phenacetin	160 mg.

Spotting Solvent: Ethanol 95 per cent

Developing Solvent: Dioxane:Benzene: 25 per cent Ammonia (40:50:10)

Quantity applied: Amobarbital 150 µg. Phenacetin 400 µg.
Amphetamine 25 µg. A.S.A. 400 µg.

Quantity recovered: (per cent)		Amobarb.	Amphetamine	A.S.A.	Phenacetin
Assay number	1.	93.9	102.3	94.6	98.3
	2.	97.0	104.3	97.2	94.9
	3.	100.0	103.8	100.0	94.6
	4.	93.5	101.9	94.6	93.8
	5.	100.0	98.2	94.7	95.9
	6.	103.3	95.7	92.5	94.9
	7.	100.0	104.3	92.1	93.0
	8.	103.3	100.0	100.0	101.9
	9.	97.1	97.8	102.6	92.4
	10.	103.2	98.0	97.1	104.1
Mean		99.1	100.6	96.5	96.4
Standard Deviation		± 3.5	± 2.9	± 3.3	± 3.7

Minimum detectable quantity: Amobarb 40 µg. Amphetamine 5 µg. Phenacetin 50 µg. A.S.A. 50 µg.

Minimum assayable quantity: Amobarb 50 µg. Amphetamine 10 µg. Phenacetin 80 µg. A.S.A. 80 µg.

Maximum assayable quantity: 400 µg. and over for Phenacetin and A.S.A.
300 µg. for Amphetamine and Amobarb.

Instrument Settings:

a. Response: 5

b. Chart Speed: 2 inches per minute

c. Filter: A.S.A. and Phenacetin-#485 Blue

Total Analysis Time: 7 hours for 10 determinations

Table XIX

Quantitative Results for 217 - Mep

Composition:

Acetylsalicylic acid	200 mg.
Phenacetin	150 mg.
Caffeine Citrate	30 mg.
Meprobamate	200 mg.

Spotting Solvent: Ethanol 95 per cent

Developing Solvent: Dioxane:Benzene: 25 per cent Ammonia (40:50:10)

Quantity applied:

A.S.A.	200 µg.
Phenacetin	150 µg.
Caffeine	60 µg.
Meprobamate	200 µg.

Quantity recovered: (per cent)		A.S.A.	Phenacetin	Caffeine	Meprobamate
Assay number	1.	92.3	93.8	96.4	95.4
	2.	90.9	100.0	94.3	97.8
	3.	92.3	89.5	100.0	96.4
	4.	90.0	94.6	95.2	101.3
	5.	95.0	97.3	100.0	100.0
	6.	100.0	97.6	96.9	101.7
	7.	92.0	91.1	97.2	90.7
	8.	92.3	90.2	91.2	97.5
	9.	96.2	91.1	96.6	91.5
	10.	96.7	93.2	97.8	96.6
Mean		93.8	93.8	96.6	96.9
Standard Deviation		± 3.0	± 3.4	± 2.5	± 3.5

Minimum detectable quantity: A.S.A. and Phenacetin 50 µg.
Caffeine Citrate 10 µg.

Minimum assayable quantity: A.S.A. and Phenacetin 80-100 µg.
Caffeine Citrate 30 µg.

Maximum assayable quantity: A.S.A. and Phenacetin 300 µg.
Caffeine Citrate 300 µg.
Meprobamate 1600 µg.

Instrument Settings:

a. Response: 5
b. Chart Speed: 2 inches per minute
c. Filter: #485 for Phenacetin and A.S.A.

Total Analysis Time: 6 1/2 hours for 10 determinations

Table XX

Quantitative Results for Triple Sulfas Tablets

Composition:

Sulfamethazine	167 mg.
Sulfadiazine	167 mg.
Sulfamerazine	167 mg.

Spotting Solvent: Ethanol 95 per cent

Developing Solvent: Dioxane:Benzene: 25 per cent Ammonia (40:50:10)

Quantity applied: 1 µg. of each.

Quantity recovered: (per cent)

		Sulfamethazine	Sulfadiazine	Sulfamerazine
Assay number	1.	93.9	94.1	91.6
	2.	104.7	91.3	100.0
	3.	91.2	95.7	105.3
	4.	97.6	92.3	92.3
	5.	94.7	95.8	92.9
	6.	100.0	90.6	100.0
	7.	95.7	95.5	94.7
	8.	88.9	100.0	100.0
	9.	104.2	94.7	91.3
	10.	94.7	95.2	95.0
Mean		96.6	94.5	96.3
Standard Deviation		± 4.9	± 2.7	± 3.8

Minimum detectable quantity: 0.2 µg. of each

Minimum assayable quantity: 0.5 µg. of each

Maximum assayable quantity: 2.5 µg. of each

Instrument Settings:

a. Response: 5

b. Chart Speed: 2 inches per minute

c. Filter: none

Total Analysis Time: 6 hours for 10 determinations

Table XXI

Effect of Developing Solvent on Spot Area

Drug	Spot Size			
	Before Development		After Development	
	Dimensions (mm.)	Approx. Area (sq. mm.)	Dimensions (mm.)	Approx. Area (sq. mm.)
d-Amphetamine sulfate	7 x 7	49	7 x 10	70
Meprobamate	7 x 7	49	12 x 22	264
Prochlorperazine	7 x 7	49	7 x 12	84
Amobarbital	7 x 7	49	12 x 15	180
Butabarbital	7 x 7	49	10 x 14	140
Pentobarbital	8 x 8	64	14 x 18	252
Methamphetamine HCl	8 x 8	64	8 x 14	112
Phenobarbital	8 x 8	64	11 x 16	176
Carbromal	5 x 5	25	10 x 14	140
Acetylsalicylic acid	7 x 7	49	8 x 8	64
Phenacetin	7 x 7	49	12 x 18	216
dl-Amphetamine	7 x 7	49	8 x 13	104
Ephedrine	8 x 8	64	9 x 14	126
Theophylline	8 x 8	64	8 x 9	72
Caffeine citrate	8 x 8	64	8 x 9	72
Sulfamerazine	3 x 3	9	7 x 6	42